ADIPOSE TRIGLYCERIDE LIPASE MEDIATED REGULATION OF SIRTUIN1 AND LIPOPHAGY

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AISHWARYA SATHYANARAYAN

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ABSTRACT

An over-accumulation of triacylglycerol (TAG) within the liver results in non-alcoholic fatty liver disease (NAFLD), which may progress to more severe states including cirrhosis and fibrosis, finally leading to liver injury and death. In most cases, the etiology of NAFLD is not completely understood but has been primarily linked to the occurrence of insulin resistance, which often accompanies obesity, dyslipidemia and Type 2 Diabetes. Previously, our laboratory discovered that adipose triglyceride lipase (ATGL) catalyzes TAG hydrolysis in the liver and partitions the hydrolyzed fatty acids to oxidative pathways. Additionally, ATGL promotes fatty acid oxidation in part through its induction of peroxisome proliferator activated receptor-α (PPAR-α) and its co-activator PPAR-γ coactivator-1α (PGC-1α) signaling. The objective of this work is to further elucidate the mechanisms underlying ATGL's effects on lipid metabolism and signaling. Herein, we show that ATGL activates the protein deacetylase sirtuin 1 (SIRT1) which subsequently deacetylates and activates PGC1- α and PPAR-α to promote transcriptional regulation of mitochondrial biogenesis and FA oxidation. ATGL preferentially cleaves FA oleate from TAG to induce SIRT1 enzyme activity in a dose-dependent manner; other FA species had no effect. Moreover, ATGL requires SIRT1 for the induction of PGC-1α/PPAR-α target genes and oxidative metabolism. ATGL also mediates the effect of β-adrenergic signaling on SIRT1 activity and PGC-1α and PPAR-α target gene expression. This work is the first to identify an endogenous activator of SIRT1 that couples β-adrenergic

signaling and lipolysis activation to enhanced transcriptional regulation of oxidative metabolism.

In addition to increased SIRT1 activity and downstream oxidative metabolism, our research reveals that ATGL activation induces a cellular recycling process called autophagy. In particular, ATGL requires functional autophagy to mediate its effects on lipid depletion in a SIRT1 dependent manner. In addition, ATGL requires SIRT1 to exert its effects on TAG turnover via autophagy. In addition, we show that ATGL mediates FA secretion via the lysosome, as confirmed in both *in vitro* and *in vivo* models, in response to fasting. Interestingly, these effluxed FAs undergo a reuptake process before being oxidized or reesterified into existing TAG depots. Overall, these data challenge the current dogma of hepatic lipid metabolism and advance our understanding of lipid droplet biology.

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CHAPTER 1

The Role of Hepatic Lipolysis in the regulation of Lipophagy and subsequent mitigation of Fatty Liver Disease

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Hepatic lipid metabolism and Non-Alcoholic Fatty Liver disease (NAFLD)

The liver is an integral organ involved in lipid metabolism and is an important contributor to both the synthesis, uptake and oxidation of fatty acids (FAs). FAs serve as the building blocks for more complex lipids and, when stored as triacylglycerol (TAG), represent the major storage of energy. Hepatic FAs are primarily obtained through *de novo* lipogenesis, lipoprotein remnant uptake and adipose tissue derived plasma non-esterified fatty acids (NEFA) (Nguyen et al., 2008). Because the liver does not serve as a major storage depot for fat, concentration of hepatic TAGs is low under normal physiological conditions.

Despite the low concentration, there is considerable trafficking of both TAGs and FAs in and out of the liver in response to a fed or fasted state (Kawano and Cohen, 2013). Hepatic TAG content is balanced through processes involved in FA uptake, lipogenesis, lipolysis, oxidation and very low density lipoprotein (VLDL) export (Nguyen et al., 2008). The liver takes up serum NEFAs, which enter the cells via transporters such as FA transport proteins (FATP) or clusters of differentiation 36 (CD36) receptors. NEFAs are subsequently activated by a member from the families of acyl-CoA synthetases (ACSLs) or FATPs (Nguyen et al., 2008). Several isoforms of ACSL have been identified and each plays a significant role in determining the fate of a particular acyl-CoA by channeling the species towards lipid synthesis or oxidation (Nguyen et al., 2008).

Once excess serum free FAs are taken up by hepatocytes and, they are stored as TAG within lipid droplets. Over-accumulation of lipid droplets within hepatocytes eventually results in hepatic steatosis, which may develop as a consequence of reduced disposal of FAs via decreased β-oxidation, very low-density lipoprotein secretion (VLDL), or enhanced uptake or synthesis of FA (Kawano and Cohen, 2013). The most common liver disease is non-alcoholic fatty liver disease (NAFLD), which may progress from a mild hepatic steatosis to more severe states including cirrhosis and fibrosis, eventually resulting in hepatocyte injury and death (Fabbrini et al., 2010; Yoneda et al., 2008)

Despite the severe consequences of NAFLD, in most cases, the etiology of NAFLD is not completely understood. It has been primarily linked to the occurrence of insulin resistance that often accompanies obesity, dyslipidemia and Type 2 Diabetes (Angulo, 2002; McCullough, 2004). NAFLD is frequently characterized by increased hepatic TAG accumulation in the absence of significant alcohol consumption and has been associated with numerous metabolic diseases including insulin resistance, Type 2 Diabetes and obesity (Utzschneider and Kahn, 2006). Specifically, insulin resistance in the adipose tissue results in increased TAG breakdown and release of NEFA, which results in increased hepatic uptake. This metabolic state also results in higher levels of circulating insulin, which promotes increased hepatic TAG synthesis and decreased mitochondrial fatty acid oxidation (Angulo, 2002). Other relevant causes that could contribute to NAFLD and cirrhosis include nutritional status of

the individual, drug-toxicity, hepatitis C virus infection and inflammatory disorders such as Inflammatory Bowel Syndrome and Crohn's disease (Cave et al., 2007; McCullough, 2004).

NAFLD is present in approximately one-third of the general population (Charlton et al., 2011; Francque et al., 2016). The prevalence is higher in obese populations with BMI $\geq$ 30, and there is an increasing incidence of non-alcoholic steatohepatitis (NASH) in morbidly obese individuals with BMI $\geq$ 40 (Dixon et al., 2001; García-Monzón et al., 2000). Given the widespread prevalence and debilitating consequences of NAFLD to other metabolic disorders, it is of paramount importance to further study its pathogenesis.

TAG hydrolysis- discovery of ATGL and its regulation

The majority of scientific literature has focused on the role of lipid synthesis in the pathophysiology of NAFLD. Several recent advances have looked at the contribution of TAG catabolism to NAFLD (Jha et al., 2013; Ong et al., 2011). Lipolysis is a critical physiological process involved in breaking down TAG to release free fatty acids and glycerol and is primarily governed by the energy status of the body.

In the fed-state when the body is replete with energy resources, insulin inhibits TAG catabolism and promotes its synthesis (Klop et al., 2013). During fasting, the body relies on its stored energy reserves such as TAG depots and lipolysis is initiated via catecholamine mediated β-adrenergic stimulation. Hepatic

TAG hydrolysis primarily serves to provide FA substrates for mitochondrial β-oxidation, signaling, and VLDL assembly for TAG export (Quiroga and Lehner, 2012). On the other hand, white adipose tissue (WAT) exhibits the highest lipolytic activity and is responsible for releasing FAs into circulation during times of starvation/fasting.

Historically, lipolysis within the WAT was believed to be catalyzed by two major lipases, namely hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL). HSL has catalytic activities towards both TAG and diacylglycerol (DAG) (Quiroga and Lehner, 2012). Thus, HSL hydrolyzes TAG to yield DAG and further breaks DAG to obtain a monoacylglyceride (MAG) moiety which is eventually hydrolyzed by MGL to liberate the final FA, leading to glycerol release. However, the hypothesis that HSL was the major lipase catalyzing the breakdown of TAG and DAG (Small et al., 1989; Yeaman, 1990) in most tissues including adipose tissue, skeletal muscles and the liver was questioned when genetic ablation of HSL failed to eliminate lipolysis in adipocytes (Lafontan and Langin, 2009; Osuga et al., 2000). This paved way to the discovery of a new lipase by three separate scientific groups in 2004, and this enzyme was termed adipose triglyceride lipase (ATGL) (Zimmermann et al., 2004), desnutrin (Villena et al., 2004) or phospholipase A2 (Jenkins et al., 2004).

ATGL selectively performs the first step in TAG hydrolysis, yielding DAG and free FAs (Zimmermann et al., 2004). Phylogenetic ancestral analysis revealed ATGL to contain a patatin-like phospholipase (PNPLA) domain, and it was thus

classified under a group of five phospholipases PNPLA 1-5 (Rydel et al., 2003). This group of enzymes contains a patatin-domain that encodes the major glycoprotein in potatoes with known DAG and MAG activity but not specific TAG hydrolase activity (Wilson et al., 2006; Zechner et al., 2005). However, ATGL was found to exhibit minimal or no activity to other lipid species such as DAG, MAG, and acylesters like retinyl esters and cholesteroyl esters (Zimmermann et al., 2004).

The human and mouse genes for ATGL encode proteins with 504 and 486 residues, respectively, and share 84% amino acid identity. The predominant regions of low sequence conservation are clustered around amino acid residue 260 and the C-terminal end of the protein (Lass et al., 2011). Sequence similarities predict that the N-terminal half of ATGL is an α/β-fold hydrolase fold protein, common to a number of hydrolytic enzymes and belonging to the superfamily of patatin-like phospholipases (Rydel et al., 2003; Wilson et al., 2006). It has been postulated that a hydrophobic stretch within the C-terminus represents a lipid binding region that is involved in regulating enzyme activity (Kobayashi et al., 2008; Schweiger et al., 2008). Additionally, human ATGL can be phosphorylated on two sites, serine 404 and serine 428, though these sites are not modified by the classical lipolytic signal protein kinase A (Bartz et al., 2007; Zimmermann et al., 2004). Furthermore, recent evidence indicates that adipose-specific AMP-activated protein kinase (AMPK) ablation has defective

phosphorylation of serine 406, lowering basal lipolysis in adipose tissue (Kim et al., 2016)

Further elucidation of the physiological function of ATGL was obtained through the ATGL knockout *(Atgl$^{(-/-)}$)* mouse model (Haemmerle et al., 2006). *Atgl$^{(-/-)}$* mice exhibited increased accumulation of neutral lipid in most tissues, particularly WAT and heart, suggesting an essential role this enzyme plays in cellular TAG catabolism. This increased lipid accumulation was accompanied by a concomitant increase in body weight gain with no changes in food intake or lean body mass measurements (Haemmerle et al., 2006). Furthermore, β-adrenergic stimulated lipolytic capacity and TAG hydrolase activity were drastically reduced in the ATGL deficient WAT, liver, and skeletal muscle (Haemmerle et al., 2006). The excessive TAG accumulation coupled with impaired TAG mobilization in the heart causes severe myopathy in cardiac muscle and defective thermogenesis in the brown adipose tissue (BAT) (Haemmerle et al., 2006). This excessive TAG accumulation in the heart results in cardiac dysfunction and premature death of ATGL-deficient mice (Haemmerle et al., 2006).

Interestingly, despite the increased TAG stores due to impaired degradation, ATGL-deficient mice had lower plasma FA, TAG, and cholesterol concentrations compared to HSL-deficient mice (Haemmerle et al., 2006). Further elucidation of hepatic ATGL has revealed this lipase promotes TAG breakdown without affecting VLDL secretion (Ong et al., 2011). More specifically,

manipulating hepatic ATGL function failed to influence TAG secretion despite significantly altering fatty acid oxidation with concomitant changes in peroxisome proliferator-activated receptor α (PPAR-α) target genes (Ong et al., 2011). Taken together, these findings indicate that ATGL is the rate-limiting enzyme that hydrolyzes TAG, while HSL primarily catalyzes degradation of DAG followed by MGL that specifically targets MAG for breakdown (Haemmerle et al., 2006; Yeaman, 1990)

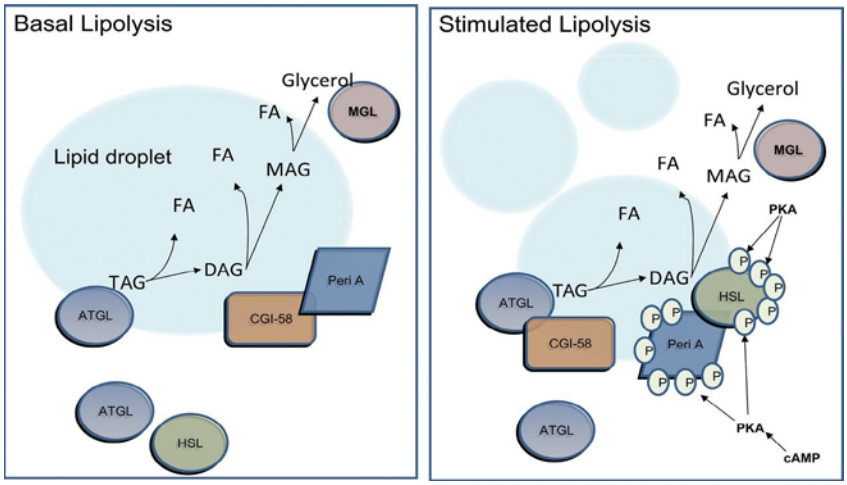
Regulation of ATGL activity is of immense research interest and has only been partially elucidated. ATGL mRNA levels are increased during fasting and repressed during re-feeding (Oliver et al., 2012; Villena et al., 2004). Additionally, insulin has also been shown to suppress lipolysis by down-regulating the expression of ATGL, which may account for some of the fasting/re-feeding regulation (Kolditz and Langin, 2010). ATGL activity is further increased by an activator protein, comparative gene identification-58 (CGI-58- also known as α/β hydrolase domain containing protein 5) (Lass et al., 2011). An important regulatory mechanism for ATGL involves the activation of the enzyme upon interaction with CGI-58 with maximal stimulation obtained at approximately a one to one stiochiometry of the enzyme and activator protein (Lass et al., 2006).

Recently, G0/G1 switch protein 2 (GOS2) protein was found to selectively inhibit ATGL (Yang et al., 2010). The mechanistic involvement of GOS2 in the cell-cycle remains unclear. However, it has been found to be expressed in cells transitioning from the G_0 to G_1 phase of the cell cycle (Russell and Forsdyke,

1991). GOS2 has been shown to directly interact with the N-terminal patatin domain of ATGL and this interaction does not directly compete with the binding of CGI-58, which also interacts with the N-terminal region of ATGL (Klar et al., 1981; Russell and Forsdyke, 1991). This interaction has been further corroborated by co-immunoprecipitation experiments demonstrating that CGI-58 does not affect the interaction between GOS2 and ATGL (Yang et al., 2010). Despite sharing the N-terminus of ATGL as a binding site, the above observations suggest that GOS2 and CGI-58 do not compete in the regulation of ATGL activity. ATGL has also been reported to be a receptor of pigment epithelium-derived factor (PEDF), a multifunctional glycoprotein involved in neuronal survival and differentiation (Notari et al., 2006). PEDF binds to ATGL and increases its TAG hydrolase activity, which is supported by data showing increased lipid accumulation in PEDF-null hepatocytes compared to the controls (Chung et al., 2008). Reversal of this steatosis was observed upon PEDF restoration, highlighting the importance of PEDF is regulating ATGL activity (Chung et al., 2008).

TAG hydrolysis generates DAGs and FAs and the resulting DAG can exist in three stereochemical isoforms. ATGL-mediated lipid catabolism specifically cleaves the FA at the *sn*-2 position of the TAG molecule generating a *sn*-1, 3 DAG isoform (Eichmann et al., 2012). In the presence of its co-activator CGI-58, ATGL additionally cleaves the FA at *sn*-1 position resulting in *sn*-2, 3 DAG (Eichmann et al., 2012). More importantly, DAG isoforms obtained through ATGL

hydrolysis are not involved in phospholipid synthesis or activation of protein kinase C, which is implicated in insulin resistance and other signaling pathways (Eichmann et al., 2012). Interestingly, diacylglycerol-*O*-acyltransferase 2 (DGAT-2), involved in DAG re-esterification to TAG, preferentially esterifies *sn*-1, 3 DAG (Eichmann et al., 2012). Thus, ATGL and DGAT-2 act coordinately in the hydrolysis and re-esterification of TAG (Eichmann et al., 2012). This stereo/regioselectivity of ATGL shows the importance of this lipase in DAG metabolism and signaling. Although DAG isoforms obtained through ATGL-derived TAG hydrolysis have been well characterized, little is known on the signaling role of specific FA species generated through the same lipolytic process.



Adapted from Matt, M.J., & Steinberg, G.R. (2008); 414(3), 313-325

Figure 1: In the basal state, ATGL is localized to the LD and not in proximity with CGI-58. Upon stimulus, phosphorylation of perilipin dissociates CGI-58 which translocates to ATGL and promotes TAG hydrolysis.

Introduction to Sirtuins - Structure and regulation

Sirtuins are Class III NAD^+ dependent histone/protein deacetylases and/or mono-ADP-ribosyltransferases found in organisms ranging from microbes to humans. They comprise a unique group of enzymes, relying on nicotinamide adenine dinucleotide (NAD^+) as an essential cofactor linking their activity to cellular metabolic status (Kaeberlein et al., 1999). Originally found in yeast, *Sir2* was discovered as a silencing factor and has been shown to mediate the effects of calorie restriction on lifespan extension (Jeggo, 1998). In addition, *Sir2* participates in repression of transcriptional activity via gene silencing and in DNA double-strand break (DSB) repair (Tanny et al., 1999). Gene silencing is a type of DNA inactivation involved in the regulation of gene expression and a major development in the field led to the discovery of the silencing protein family of *Sir2* and its members (Brachmann et al., 1995).

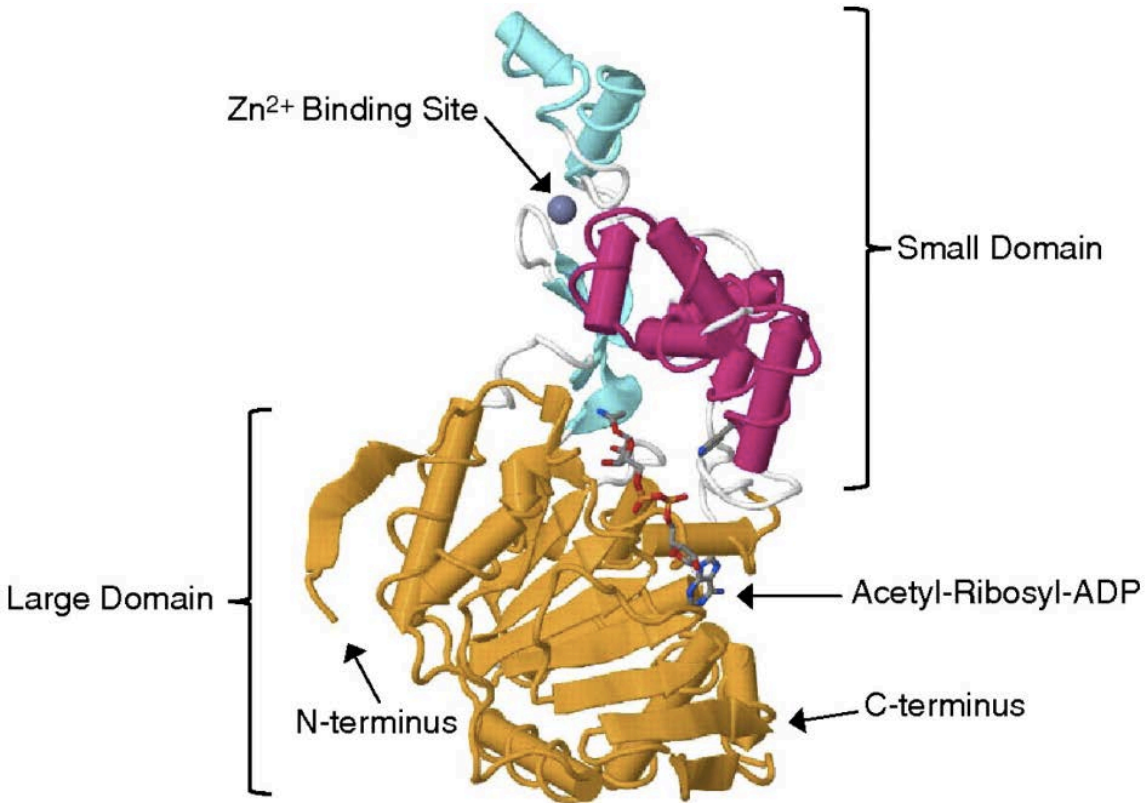
Besides their role in transcriptional silencing, yeast sirtuins have also been implicated in regulating genomic stability, cell cycle progression, life span extension, and oxidative stress (Toiber et al., 2011). Cells lacking *Sir2* have a reduced replicative life span while cells with additional *Sir2* display a much longer life span than the control wild type (Sinclair and Guarente, 1997). This lifespan extension is a consequence of hypersilencing of the rDNA, which reduces recombination and the production of extrachromosomal rDNA circles, a known cause of senescence in ageing mother cells (Bitterman et al., 2002).

Based on the homology to yeast histone deacetylases, seven mammalian homologs of *Sir2* (SIRT1 - 7) have been identified and are divided into four subclasses based on sub-cellular localization and deacetylase activity. SIRT1, 6, and 7 are mostly present in the nucleus; SIRT2 is predominantly cytoplasmic but translocates to the nucleus during the cell cycle; and SIRT3, 4, and 5 have been characterized as mitochondrial sirtuins (Finkel et al., 2009). Moreover, SIRT 1-3 have demonstrated robust deacetylase activity, whereas SIRT4 appears to function mainly as an ADP-ribosyl transferase (Imai et al., 2000). SIRT6 has both ADP-ribosyltransferase and deacetylase activity and SIRT5 has been shown to exhibit more demalonylase and desuccinylase activity than that of a deacetylase (Imai et al., 2000; Yuan and Marmorstein, 2012).

A classic sirtuin deacetylation reaction catalyzes the transfer of the acetyl group from the acetyl lysine of proteins to NAD^+ resulting in 2′-*O*-acetyl-ADP-ribose and free nicotinamide (Kaeberlein et al., 1999). Cleavage of the glycosidic bond between nicotinamide and ribose of NAD^+ during the sirtuin mediated deacetylation results in the transfer of an acetyl group on the acetylated lysine residue of the target peptide to the ribose moiety of ADP-ribose. The nicotinamide by-product is a non-competitive inhibitor of sirtuins and inhibits enzyme activity through a base exchange process, resulting in an intermediate β-NAD^+ which blocks further deacetylation (Du et al., 2011; Jackson et al., 2003).

Several crystallography studies of sirtuin structures have provided deeper insight into its molecular mechanisms of substrate specificity, regulation and

catalysis. Primary sequence alignment of sirtuins has revealed that they share a highly conserved catalytic core with sequentially variant N- and C- terminal segments of different lengths. This conserved catalytic core domain contains a Rossmann fold domain specific for NAD^+ binding, a helical module consisting of three to four helices, and a variable small zinc-binding ribbon module (Chakrabarty and Balaram, 2010). The Rossmann fold further links the two modules, resulting in a hydrophobic cleft formation which serves as the binding site for the substrate (preferably a thioacetyllysine peptide) and NAD^+ on opposite sides thus leading to catalysis (Chakrabarty and Balaram, 2010). The zinc ion does not participate in the deacetylation process but plays an integral role in maintaining structure. Mutations leading to loss of the zinc have been shown to abolish deacetylase activity due to collapses within the structure (Levine and Klionsky, 2004).



Adapted from Nogueiras et.al. 2012. APS 10.1152

Figure 2: Crystal structure of *Sir2* with an acetyl-ribosyl-ADP intermediate. The zinc binding site is highlighted in cyan and the Rossmann fold like NAD^+ binding site is highlighted in orange.

SIRT1 is the mammalian ortholog of yeast *Sir2*. The importance of this deacetylase in a growing number of cellular processes resulted in the synthesis of selective activators and inhibitors to further dissect its role. Howitz et al. was the first to identify SIRT1 activators through a high-throughput screening using a commercial fluorescence deacetylation kit (Borra et al., 2005). Resveratrol, a polyphenol found in red wine was identified as the most potent activator and was

shown to decrease the K_m of the substrate and NAD^+ with no changes in the V_{max} of the reaction, suggesting it was an allosteric regulator of SIRT1 (Borra et al., 2005). However, two independent reports attributed the *in vitro* effects of resveratrol-mediated SIRT1 catalyzed deacetylation as a substrate artifact (Kaeberlein et al., 2005; Pacholec et al., 2010). Resveratrol was shown to increase the affinity and deacetylation of the fluorophore-peptide with no influence on the peptide in the absence of the fluorophore group (Kaeberlein et al., 2005; Pacholec et al., 2010). This dispute eventually resulted in the conclusion that resveratrol increased SIRT1 activity, though not necessarily through direct activation (Pan et al., 2012).

A study in 2011 looked at truncations in the N- and C- terminal segments of SIRT1 to play a role in modulating the deacetylase activity (Hubbard et al., 2013). It was thus identified that the N-terminus was required for maximum catalytic activity while the conserved catalytic core domain demonstrated weak activity independently (Hubbard et al., 2013). Furthermore, the C-terminus was shown to influence the activity of established sirtuin inhibitors, known to function through the catalytic core domain (Hubbard et al., 2013). Despite the ongoing debate on the legitimacy of mechanism of action, the pharmacological relevance of SIRT1 activation and the mechanism of action of STACs are still of great interest.

Role of SIRT1 in energy metabolism

NAD^+ dependent SIRT1 activity is important in the regulation of cellular and systemic energy homeostasis because it can sense nutrient availability and connect cellular energy status to various transcriptional outcomes. Studies looking at calorie restriction, fasting, and exercise show increases in cellular NAD^+ levels in mammalian tissues with a corresponding increase in SIRT1 deacetylase activity (Guarente, 2007; Houtkooper et al., 2012; Murugesan et al., 2013). Additionally, activators of AMPK, a major cellular energy sensor, have been shown to increase NAD^+ levels thereby increasing SIRT1 activity (Cantó et al., 2009).

Once activated, SIRT1 demonstrates affinity towards a broad range of histone and non-histone peptide substrates, including transcription factors involved in stress response and metabolism pathways (Cantó et al., 2009). Prolonged fasting has been shown to increase SIRT1-mediated deacetylation of key regulators in gluconeogenesis, fatty acid oxidation and mitochondrial biogenesis including forkhead box O-1 (FOXO-1) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), thus increasing their transcriptional activity (Brunet et al., 2004; Rodgers et al., 2005). The cyclic AMP- responsive element binding protein (CREB) positively induces SIRT1 gene transcription during fasting. Conversely, feeding decreases SIRT1 activity via increased carbohydrate response element binding protein (ChREBP) translocation to the nucleus and binding the SIRT1 promoter, at a site that

overlaps CREB binding, thereby repressing gene transcription (Noriega et al., 2011).

It has also been demonstrated that pathological conditions such as fatty liver in obesity decreases deacetylase activity of SIRT1 (Coste et al., 2008). Decreased levels of intracellular NAD^+ in fatty livers of obese mice also contribute to reduced SIRT1 deacetylase activity (Yoshino et al., 2011). Additionally, high fat diet-induced metabolic stress increases the interaction between SIRT1 and its endogenous inhibitor, deleted in breast cancer 1 (DBC1), resulting in decreased deacetylase activity (Escande et al., 2010). Animal studies using STACs have revealed SIRT1 activation to increase mitochondrial content, alleviate insulin resistance and improve overall metabolic profiles in a diet-induced obese model (Baur et al., 2006; Feige et al., 2008; Lagouge et al., 2006). Mice over-expressing SIRT1 are resistant to weight gain and insulin resistance with amelioration in glucose intolerance when fed a high fat diet in comparison with wild-type control mice (Pfluger et al., 2008).

Consistent with these gain-of-function studies, SIRT1 loss-of-function in mice results in detrimental metabolic effects. A liver specific SIRT1 knockout (SIRT1 LKO) mouse model develops hepatic steatosis with altered fatty acid metabolism when fed a high fat diet (Purushotham et al., 2009). SIRT1 LKO mice also show impairments in insulin signaling via the mTORC2/Akt pathway, leading to oxidative stress, hyperglycemia, and insulin resistance (R.-H. Wang et al., 2011). Fasting further induces SIRT1 deacetylation and down-regulation of a key

lipogenic activator, sterol regulatory element binding protein 1c (SREBP-1c) via increased proteosomal degradation and ubiquitination (Ponugoti et al., 2010).

It has been demonstrated that SIRT1 positively regulates a main transcription factor regulating hepatic lipid metabolism, PPAR-α, and hepatocyte-specific deletion of SIRT1 results in impaired PPAR-α signaling (Purushotham et al., 2009). Furthermore, SIRT1 increases the transcriptional activation of PGC-1α via deacetylation to increase β-oxidation of fatty acids, leading to lower hepatic TAG levels (Purushotham et al., 2009; Rodgers et al., 2008). However, it still remains unknown whether SIRT1 deacetylases PPAR-α, despite scientific evidence showing SIRT1 regulation of its isoform, PPAR-γ, in the adipose tissue (Qiang et al., 2012).

Autophagy: A fasting response similar to lipolysis

Autophagy is an intracellular lysosomal-mediated degradative pathway targeting cytosolic components such as organelles and proteins to be self-digested thereby maintaining cellular homeostasis. During the autophagic process, a single membrane structure surrounds portions of the cytoplasm and organelles and fuse to form a double-membrane autophagosome (Howitz et al., 2003). The outcome of autophagy is the complete dissociation of the substrate into its essential components, such as proteins to amino acids and TAG to free FAs. The best understood role of autophagy is cellular housekeeping, but in addition autophagy participates in processes involved in growth control, cell

defense adaption and cell differentiation (Hennig and Neufeld; Meléndez et al., 2003).

Three forms of autophagy have been identified in mammalian cells-macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (Howitz et al., 2003). Macro- and microautophagy are similar processes except in macroautophagy, the components are sequestered within an autophagosome, which fuses with a lysosome to form an autolysosome. In microautophagy, sequestration occurs directly within the lysosome. CMA is a chaperone-dependent selection of cytosolic components that are targeted to the lysosomal membrane for degradation (Howitz et al., 2003). Additionally, macroautophagy can exist in selective forms within the cell such as mitophagy (resulting in mitochondrial turnover) and lipophagy (lipid turnover) to name a few (Czaja et al., 2013).

Certain features of the hepatocytes and the liver, such as its regenerative capacity, make it an organ dependent on autophagy. Impaired autophagy can result in accumulation of toxic products within hepatocytes, thereby increasing oxidative stress through damaged organelles and eventually culminating in extensive cellular injury (Czaja et al., 2013). Additionally, the liver is an important metabolic organ and during nutrient depletion, autophagic degradation within hepatocytes recycles cellular components to provide substrates for ATP generation (Lum et al., 2005). The above described function of autophagy in the liver suggests a mechanism by which autophagy may modulate excessive

storage of TAG in NAFLD. Alternatively, aging could promote NAFLD development through decreased autophagic capacity of the cell (Czaja et al., 2013). Despite the tremendous scientific advances exploring the importance of hepatic autophagy in cell homeostasis, further investigations are required to elucidate the importance of this process in hepatic pathological conditions, specifically NAFLD.

Lipophagy: LD specific catabolism via autophagy

In the recent past, an alternate pathway of lipid degradation has garnered attention wherein the cell breaks down lipid via the lysosome (Kaushik and Cuervo, 2015; Schroeder et al., 2015). This process is termed lipophagy and it serves as a mechanism to regulate intracellular lipid stores and control energy homeostasis through breakdown of TAG. In this form of lipid metabolism, TAG in LDs is hydrolyzed by acid hydrolases within the lysosome. The primary lipase in the lysosome is lysosomal acid lipase (LIPA) which can hydrolyze all three fatty acids present in TAG. LIPA is a key enzyme involved in intracellular lipid metabolism and FA trafficking and is responsible for the lysosomal hydrolysis of TAG into FFAs and cholesterol (Fasano et al., 2012). Deficiency or mutations of this enzyme result in rare metabolic storage diseases, namely Wolman's disease (Baratta et al., 2015) and cholesterol ester storage disease (Kuranobu et al., 2016). The clinical manifestations of this disease include a marked reduction in LIPA activity resulting in an accumulation of cholesteryl esters and TAG within

the lysosomes in many tissues (Baratta et al., 2015; Kuranobu et al., 2016). Infants with Wolman's disease show growth retardation due to malabsorption, hepatomegaly and severe liver dysfunction, making survival beyond one year of age rare (Muntoni et al., 2013).

The consequences of LIPA dysregulation resulting in lipoprotein alterations similar to a NAFLD model indicate that both diseases share common pathophysiological mechanisms. However very few studies to date have assessed LIPA activity in patients with NAFLD and prevalence of the LIPA gene mutation is largely unknown. Further research is required to better clarify the mechanisms through which modulations in LIPA activity are associated with dyslipidemia and liver dysfunction (Baratta et al., 2015; Fasano et al., 2012)

SIRT1 mediated lipophagy

It has been shown in yeast that withdrawal of nutrients results in the formation of autophagosomes with subsequent induction of autophagy (Lum et al., 2005; Shintani and Klionsky, 2004). Calorie restriction or nutrient deprival has also been shown to induce *Sir2* (Jeggo, 1998) and SIRT1, therefore mediating the life-extending benefits of the sirtuins (Cohen et al., 2004). In this context, several studies have examined the role of SIRT1 in regulating autophagy (Lee et al., 2008; McBurney et al., 2013; Shin et al., 2013). SIRT1 also regulates several members of the transcription factor Forkhead Box-O (FoxO). Of this family, FoxO3 is a major regulator of ubiquitin-mediated proteasomal degradation and is

induced by fasting (Sandri et al., 2004). FoxO3 has also been shown to increase expression of autophagy-associated proteins such as LC3 and Bnip3 (Mammucari et al., 2007).

Recent studies also suggest that autophagy partakes in the process of lipid metabolism and this pathway is dependent on SIRT1 (Dong et al., 2013; Shibata et al., 2009; Singh et al., 2009). Genetic inhibition of the autophagy-related gene Atg7 in hepatocytes resulted in an increase in hepatic TAG content and lipid droplet accumulation (Singh et al., 2009). Additionally, exogenous lipid supplementation increases intracellular trafficking of lipids to the autophagosomes as demonstrated through fluorescence measurements and electron microscopy experiments (Singh et al., 2009). This increased movement of lipids to the autophagosome occurs during nutrient deprivation and this was corroborated through enhanced association of lipid droplets and autophagosomal protein LC3 during starvation (Singh et al., 2009). Though this mechanism is still unclear, it has been suggested that several lipid droplet proteins could mediate the interaction with the autophagosome (Czaja et al., 2013). Thus, the rate limiting enzyme in TAG hydrolysis, ATGL, and its regulators might be crucial in regulating lipophagy in the liver and it would be interesting to further elucidate whether SIRT1 mediates this mechanism.

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CHAPTER 2

ATGL-Catalyzed Lipolysis Regulates SIRT1 to Control PGC-1α/PPAR-α Signaling

Aishwarya Sathyanarayan, Salmaan A. Khan and Douglas G. Mashek

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SUMMARY

Ample research has shown that adipose triacylglycerol lipase (ATGL) increases the activity of the nuclear receptor peroxisome proliferator-activated receptor-α (PPAR-α), a PPAR-γ coactivator-1α (PGC-1α) binding partner, to promote fatty acid oxidation. Since fatty acids bind and activate PPAR-α, it has been presumed that fatty acids derived from ATGL-catalyzed lipolysis act as PPAR-α ligands. However, our laboratory has shown that administration of fenofibrate, a PPAR-α ligand, was unable to rescue PPAR-α target gene expression in the absence of ATGL suggesting an alternate mechanism must exist. Sirtuin 1 (SIRT1) is a NAD^+ dependent protein deacetylase that regulates deacetylation of a host of target proteins including PGC-1α, a transcriptional co-regulator that binds numerous transcription factors to promote oxidative metabolism and mitochondrial biogenesis. Thus, given the importance of SIRT1 in regulating PGC-1α and subsequently PPAR-α, we investigated if ATGL manipulations altered SIRT1 activity as a mechanism to regulate the expression of oxidative genes. We show that SIRT1 deacetylase activity is positively regulated by ATGL to promote PGC-1α signaling. In addition, ATGL mediates the effects of β-adrenergic signaling on SIRT1 activity and downstream PGC-1α/PPAR-α target gene expression. Furthermore, we demonstrate that SIRT1 is required for the induction of PGC-1α/PPAR-α target genes and oxidative metabolism in response to increased ATGL-mediated lipolysis. We identify that oleate, produced from lipolysis directly activates SIRT1. Taken together, our work

identifies a novel signaling axis involving β-adrenergic signaling, ATGL-catalyzed oleate and SIRT1 activation that governs transcriptional regulation of oxidative metabolism and mitochondrial biogenesis.

INTRODUCTION

SIRT1, first discovered in yeast, is a NAD^+ dependent protein deacetylase and metabolic regulator that maintains cellular homeostasis through changes in energy metabolism. Activated upon calorie restriction or exercise, SIRT1 deacetylates a host of target proteins to promote oxidative metabolism, stress resistance and reduce cellular damage. In response to fasting or exercise induced β-adrenergic signaling, the cAMP/protein kinase A (PKA) pathway activates SIRT1 to promote downstream oxidative metabolism (Gerhart-Hines et al., n.d.).

Among the several SIRT1 targets, PGC-1α is a principal target that upon deacetylation and activation, binds numerous transcription factors including PPAR-α, which is highly expressed in the liver and involved in mitochondrial biogenesis and oxidative metabolism. Furthermore, SIRT1 directly binds PPAR-α and potentiates PGC-1α/PPAR-α interactions (Purushotham et al., 2009). Hepatic specific SIRT1 knockdown is concomitant with increased fat accumulation and inflammation (Purushotham et al., 2009; Xu et al., 2010).

Our laboratory and others have shown that ATGL is a major hepatic lipase that increases the activity of PPAR-α and its target genes to promote fatty acid oxidation (Ong et al., 2011; Obrowsky et al., 2012; Haemmerle et al., 2011; Ahmadian et al., n.d.). Fatty acids bind and activate PPAR-α (Forman et al., 1997; Kliewer et al., 1994); therefore, it has been presumed that fatty acids derived from ATGL-catalyzed lipolysis act as PPAR-α ligands. However,

administration of a PPAR-α agonist to mice with ablated hepatic ATGL was unable to normalize oxidative gene expression (Ong et al., 2011), suggesting that a more complex mechanism exists that links ATGL to PPAR-α regulation. Given the importance of SIRT1 in regulating PPAR-α (Purushotham et al., 2009), we tested if manipulating ATGL alters SIRT1 activity to regulate PPAR-α activity and subsequent oxidative metabolism. Herein, we characterize a novel signaling axis involving β-adrenergic signaling, ATGL-catalyzed lipolysis, and SIRT1 activation that governs hepatic oxidative metabolism. Further, we show that oleate generated specifically by ATGL-catalyzed lipolysis directly activates SIRT1. These studies identify for the first time a non-substrate endogenous metabolite that positively regulates SIRT1 activity.

EXPERIMENTAL METHODS

SIRT1 fluorometric kinetic assay

SIRT1 activity was measured with a fluorometric kinetic assay kit (Enzo Life Sciences catalogue number: BML-AK555-001) according to the manufacturers protocol. For cellular assays using the fluorometric kinetic assays, crude nuclear protein was isolated by sucrose gradient centrifugation, and activity was normalized to protein content. One U of the recombinant SIRT1 (Enzo Life Sciences) was used according to kit protocol with 0.5 mM acetylated fluor peptide, and 10 mM NAD^+. This enzyme-substrate complex was incubated in the presence of activator for 15 minutes and the reaction quenched by the addition of a developer II reagent. The developer II was incubated for a further 45 min before reading with an excitation wavelength of 360 nm and emission at 460 nm. The final mixture was incubated for 45 min before reading the plate (Bio-Tek Synnergy HT Microplate Reader) at an excitation wavelength of 360 nm and emission at 460 nm. SIRT1 activity is expressed as percentage change over control.

Fatty acid activation assay: 1 mM stocks of different fatty acids were prepared in pure EtOH and dried down using a nitrogen evaporator. Once completely dried, the lipids were reconstituted and conjugated with 2.1 mM BSA and the final volume was made up using water. Fluorometric SIRT1 assay was performed as

detailed above; the substrate and developer II was prepared as per kit instructions.

SIRT1 recycling assay.

SIRT1 reactions were carried out in a final volume of 200 µL per well in a flat-bottom clear 96-well plate. Assay mixtures contained different concentrations of AcH3 as described in the Figure 2A, 200 µM NAD^+, 0.2 mM NAD(P)H, 1 mM DTT, 3.3 mM α-ketoglutarate, 2 µM PncA (nicotinamidase), 3 units of glutamate dehydrogenase from bovine liver, 1 µM SIRT1 in 20 mM potassium phosphate at pH 7.5. All assay components except SIRT1 or NAD^+ were preincubated at 25°C for 5 min or until absorbance at 340 nm stabilized and the reaction was initiated by addition of SIRT1 or NAD^+. The rates were analyzed continuously for 10, 20, 30, 60, 90 and 120 min by measuring NAD(P)H consumption at 340 nm. Rates were determined from the slopes of the initial linear portion of each curve using an extinction coefficient for NAD(P)H of 6.22 mM^{-1} cm^{-1}. The background rates of reactions lacking either SIRT1 or NAD^+ were subtracted from the initial velocities of the SIRT1-catalyzed reactions. Data is presented as units/min (U/min) and 1 unit is defined as change in absorbance at 340nm over change in time.

PPAR-α and PGC-1α Reporter Assay

MEFs were transfected with indicated firefly luciferase reporter plasmids (TK-MH-UASluc), control Renilla luciferase (pRLSV40), and indicated GAL4-PPAR-α or

PGC-1α constructs using Effectene Transfection Reagent (QIAGEN). For PPAR-α reporter activity, pSG5-GAL4-PPARα-LBD construct was transfected into cells. For PGC-1α reporter activity, pCMX-GAL4-PGC1α, provided by Brian Finck (Washington University in St. Louis), was transfected into cells. To raise ATGL expression, MEFs were transduced with 60 multiplicity of infection Ad-ATGL or Ad-GFP for 18 h. To inhibit SIRT1 activity, MEFs were incubated for 18 h with 10 mmol/L EX-527. Cells were stimulated with 1 mmol/L cAMP analog for 4 h. Following treatments with indicated adenovirus or drugs, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to the coexpressed Renilla luciferase activity.

RNA Isolation and RT-PCR Analysis

RNA was extracted with TRIzol from liver tissues followed by reverse transcription with SuperScript VILO cDNA Synthesis Kit (Invitrogen) to generate cDNA. Gene expression was quantified as described previously (Ong et al., 2011).

RESULTS

ATGL is required to regulate β-adrenergic induced hepatic SIRT1 activity and downstream PGC1-α /PPAR-α signaling

Since PPAR-α signaling is a major pathway affected by hepatic ablation of SIRT1, we sought to examine if SIRT1 plays a role in ATGL-mediated regulation of PPAR-α activity. Hepatic SIRT1 activity was suppressed by ~50% in mice treated with adenovirus harboring shRNA targeted to ATGL, whereas adenoviral-mediated overexpression of ATGL increased hepatic SIRT1 activity (Figure 1A). SIRT1 mRNA abundance was also reduced in response to ATGL knockdown (Figure 1B), and targets of SIRT1, PGC-1α. and FOXO1 (forkhead box protein O1), were hyperacetylated in response to ATGL knockdown (data not shown) consistent with reduced SIRT1 activity.

Since β-adrenergic signaling increases lipolysis and SIRT1 activity (Murugesan et al., 2013; Rodgers et al., 2005), we next explored the connection between ATGL and SIRT1. Pre-treatment of primary mouse hepatocytes with bromoenol lactone (BEL), a lipase inhibitor, blocked the ability of a cell-permeable cAMP analogue (8-Bromoadenosine 3′,5′-cyclic monophosphate) to stimulate SIRT1 activity (Figure 1C). Attenuating cAMP/PKA signaling with a PKA inhibitor (H89) also blocked SIRT1 activation by cAMP in primary hepatocytes (Figure 1D), suggesting that the PKA arm of the β-adrenergic signaling cascade is responsible for the observed effects. Consistent with its inhibition of SIRT1, BEL also blocked the induction of PGC-1α reporter activity in

response to cAMP in mouse embryonic fibroblasts (MEFs) (Figure 1E). Similarly, BEL blocked the induction of PPAR-α and PGC-1α and their target genes in cultured primary hepatocytes in response to cAMP (data not shown). Taken together, these findings demonstrate that ATGL-catalyzed lipolysis is required for the cAMP/PKA-mediated activation of SIRT1 and induction of PGC-1α/PPAR-α target genes.

Oleate released from ATGL-catalyzed lipolysis activates SIRT1

Based on the above data showing that ATGL regulates SIRT1 activity, we hypothesized that catabolism of triglycerides by ATGL releases specific lipolytic products or downstream metabolites that activate SIRT1. Additionally, global and liver-specific ATGL knockdown in mice leads to enrichment of oleate in liver triglyceride (Eichmann et al., 2012; Ong et al., 2011), suggesting that ATGL preferentially hydrolyzes oleate. Furthermore, it has been shown both *in vitro* and *in vivo* that with its co-activator CGI-58, ATGL shows highest affinity for the monounsaturated fatty acids palmitoleic and oleic acid at *sn*-1 and *sn-2* positions resulting in *sn-1,3* and *sn*-2, 3 DAG (Eichmann et al., 2012). Using a commercial fluorometric kinetic assay with recombinant SIRT1 and a TAMRA fluoro-peptide, we found that oleate increased SIRT1 activity in a dose-dependent manner (Figure 2A). In contrast, the saturated fatty acid palmitate, and polyunsaturated fatty acids eicosapentaenoate (EPA), docosahexaenoate (DHA), and linoleate (LA) did not increase SIRT1 activity.

We next evaluated SIRT1 enzyme kinetics in response to varying concentrations of acetylated histone H3 peptide (AcH3) in the SIRT1 recycling assay. These data revealed that oleate lowers the Km for the AcH3 peptide substrate approximately 10-fold (Figure 2B). Incubating primary hepatocytes with oleate for 60 min induced SIRT1 activity, but inhibition of lipolysis by BEL abolished this activation (Figure 2C). These data suggest that the mechanism to activate SIRT1 involves a specific pool of oleate that is generated after esterification of oleate into triglyceride and its subsequent lipolysis by ATGL.

SIRT1 is required for the effects of ATGL-catalyzed oleate on PGC-1α/PPAR-α Activity

To examine the requirement for SIRT1 in mediating the effect of ATGL-catalyzed lipolysis, we used a luciferase assay system to test PGC-1α/PPAR-α reporter activity. Increasing the ratio of oleate to palmitate exposure in MEFs and primary mouse hepatocytes synergized with cAMP to induce Gal4-PGC1-α activity in agreement with the specificity of oleate on SIRT1 activation (Figure 3A). Finally, Gal4-PGC-1α activity was stimulated by both cAMP and ATGL overexpression in MEF cells, but this activation was abolished in response to chemical inhibition of SIRT1 (Figure 3B) and in SIRT1 knockout MEFs (Figure 3C). Taken together, these data demonstrate that SIRT1 is required for ATGL-mediated induction of oxidative metabolism.

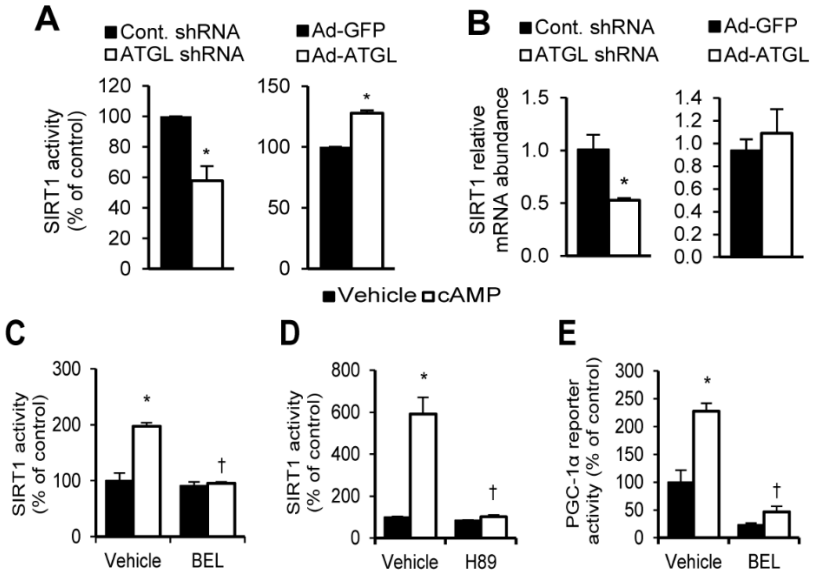

Figure 1. ATGL/lipolysis activates SIRT1. (1A&B) SIRT1 activity (relative fluorescence units (RLU) normalized to liver nuclear protein lysate) and gene expression determined from liver tissues of mice treated with the indicated adenovirues for 7 days. (1C) SIRT1 activity of primary hepatocytes treated with or without 1 mM cAMP analogue (8-Bromoadenosine 3′,5′-cyclic monophosphate) for 10 minutes, after a 1hour pretreatment with 2 μM lipase inhibitor, bromoenol lactone (BEL). (1D) SIRT1 activity as determined by deacetylase fluorometric assay of primary hepatocyte nuclear lysates. Cells were treated with or without 1mM cAMP analogue for 10 minutes, after a 1 hour pre-treatment with 30 μM protein kinase A inhibitor H89. (1E) Inhibition of ATGL blocks cAMP induction of PGC-1α reporter.

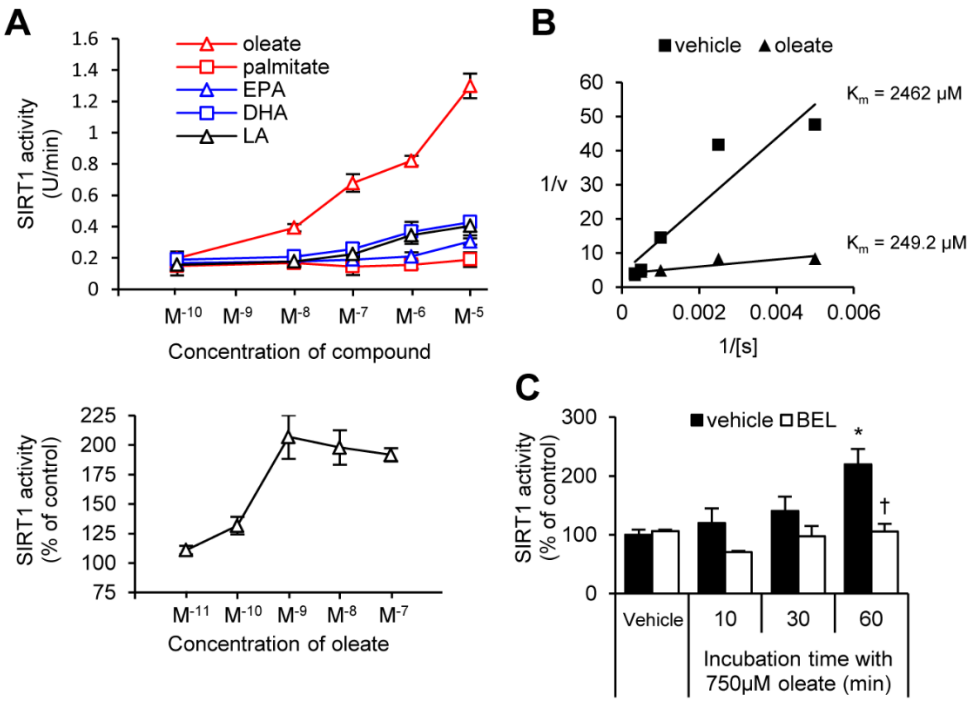

Figure 2. Oleate activates SIRT1. (2A) Dose-dependent activation of SIRT1 by oleate compared with other fatty acids. SIRT1 activity was determined by cycling assay with increasing concentrations of oleate, palmitate (PA), eicosapentaenoate (EPA), docosahexaenoate (DHA), or linoleate (LA). Increased SIRT1 activity with oleate as determined by fluorometric kinetic assay. (2B) Oleate decreases SIRT1 K_m for AcH3 substrate. (2C) Exogenous oleate requires lipolysis to activate SIRT1 in primary mouse hepatocytes. Primary mouse hepatocytes were incubated with the indicated concentration of oleate complexed to BSA, or BSA alone (vehicle) for 10, 30 or 60 min, following pre-incubation with BEL 2 µM BEL for 1 h. SIRT1 activity was determined from nuclear lysates using the fluorometric kinetic assay.

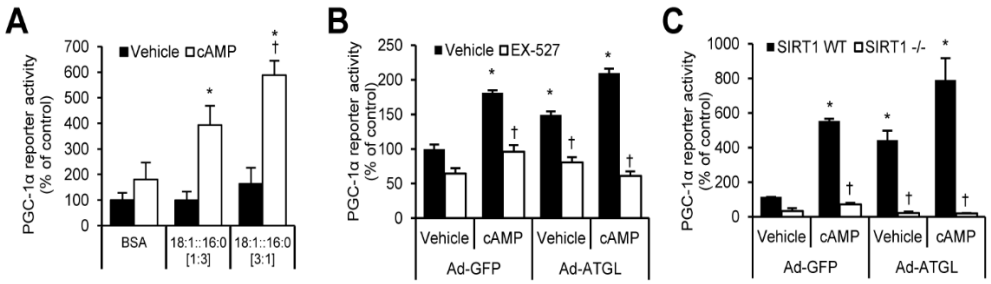

Figure 3. SIRT1 is required for the effects of ATGL on PGC-1α/PPAR-α activity.

(3A) Increasing the proportion of exogenous oleate synergizes with cAMP to induce PGC-1α reporter activity. MEFs were cultured with 400 μM total fatty acids differing in the proportion of palmitate and oleate for 16 h and dual luciferase reporter activity was subsequently measured. (3B) Chemical inhibition of SIRT1 blocks the effect of cAMP and ATGL on PGC-1α activity. Wild type MEFs were transfected with PGC-1α reporter constructs and transduced with indicated adenovirus followed by incubation with 10 μM EX527 for 18 h. Cells were then treated with or without 1mM cAMP analogue for 4 h prior to cell lysis and analysis for PGC-1α reporter activity. (3C) cAMP and ATGL activate PGC-1α reporter activity in a SIRT1 dependent manner. Wild type or SIRT1 -/- MEFs were transfected with Gal4-PGC-1α reporter constructs, transduced with indicated adenovirus, stimulated with cAMP, and reporter activity was determined.

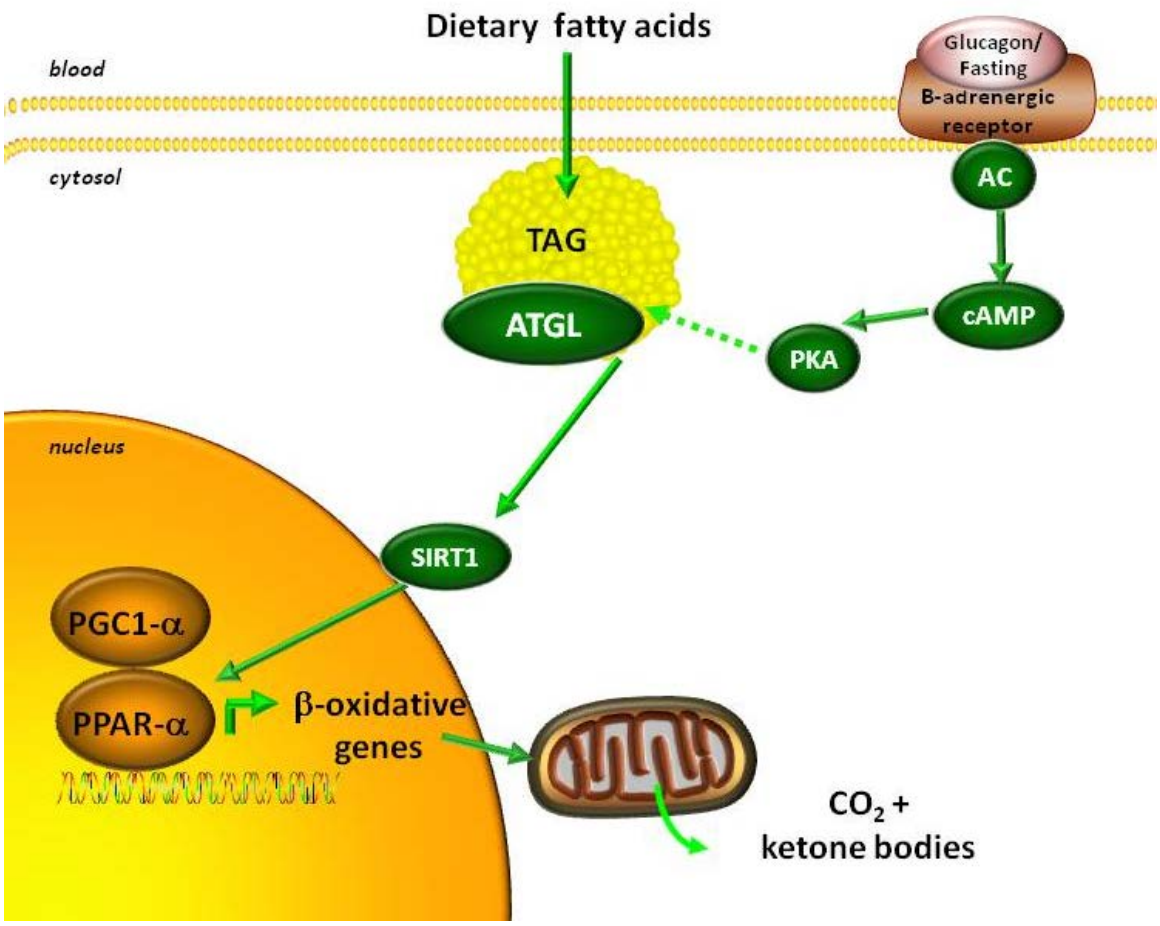

Figure 4: Summary/Model: The above data shows that β-adrenergic stimulation requires ATGL to activate SIRT1 and that oleate derived through ATGL hydrolysis, activates SIRT1 and its downstream targets. We have shown that oleate is a direct activator of SIRT1, but further studies are required to elucidate this mechanism of regulation.

DISCUSSION

These studies identify a new signaling mechanism whereby an endogenously produced metabolite directly regulates SIRT1 activity. We show that only oleate produced exclusively from lipolysis activates SIRT1 suggesting that there are inherently different pools of intracellular fatty acids with unique signaling properties. Moreover, these data provide a mechanism for the previous observation that oleate enhances the SIRT1/ PGC-1α pathway (Lim et al., 2013)

It is currently unknown how oleate regulates SIRT1. Recent publications have shown that numerous sirtuins, including SIRT1, possess long chain deacylase activity (Feldman et al., 2013; Jiang et al., 2013). Moreover, long chain fatty acids activate SIRT6 deacetylase activity, but competitively inhibit long chain deacylase activity suggesting that fatty acids bind within the SIRT6 catalytic pocket (Feldman et al., 2013). Thus, it is plausible that oleate binds within the catalytic pocket of SIRT1 to promote deacetylase activity. Additional studies are underway to further define the mechanism through which oleate facilitates SIRT1 deacetylase activity.

Numerous studies have linked lipolysis, mediated through manipulations of ATGL or other lipid droplet proteins, to changes in PPAR-α and oxidative gene expression (Ahmadian et al., n.d.; Haemmerle et al., n.d.; Obrowsky et al., n.d.; Ong et al., 2011; Sapiro et al., 2009). Because fatty acids are ligands for PPAR-α, it has been presumed that fatty acid generated from lipolysis acts as agonists for PPAR-α (Mottillo et al., 2012). However, we have shown that administration

of a PPAR-α agonist, results in a similar fold-induction of PPAR-α target genes in livers of mice treated control or ATGL shRNA adenoviruses, but was unable to normalize gene expression between the two groups (Ong et al., 2011). Consequently, our data shows that ATGL, via oleate, activates SIRT1 and provides strong evidence for an alternate signaling mechanism linking lipolysis to PPAR-α activity. Consistent with our findings, administration of a PPAR-α agonist (fenofibrate) was also unable to normalize PPAR-α target gene expression in fasted mice with ablated hepatic SIRT1 (Purushotham et al., 2009).

The discovery that resveratrol a polyphenol in red wine activates SIRT1 was thought to be the one of the several hypotheses that explain the low rates of cardiovascular disease and potential beneficial effects on the metabolic syndrome with the Mediterranean Diet (Amiot et al., 2016). However, this enthusiasm has been tempered by the observation that supraphysiological doses of resveratrol are required to elicit its effects and by data showing that the effects of resveratrol on SIRT1 are indirect and non-specific (Borra et al., 2005; Kaeberlein et al., 2005; Pacholec et al., 2010). Novel activators as well various methods to manipulate NAD^+ metabolism have since emerged as alternatives to increase SIRT1 activity and to recapitulate the effects of resveratrol (Baur, 2010). However, it remains unclear if the Mediterranean Diet activates SIRT1 independent of resveratrol. Olive oil and nuts, both high in the monounsaturated fatty acid (MUFA) oleate, are also major constituents of the Mediterranean Diet. The current study shows that ATGL-mediated hydrolysis of oleate is required for

the effects of β-adrenergic signaling on SIRT1 activity. These data suggest that factors contributing to β-adrenergic signaling such as fasting, caloric restriction or exercise are required for oleate-mediated activation of SIRT1. Thus, we speculate that the above-defined regulation of SIRT1 may be an important mechanism explaining the health benefits of the Mediterranean Lifestyle, namely adherence to the Mediterranean Diet and abundant physical activity. Ongoing studies to define the mechanism through which oleate activates SIRT1 and factors that regulate ATGL and selectivity towards oleate hydrolysis may provide novel avenues to activate SIRT1 to prevent or treat metabolic diseases and regulate lifespan.

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CHAPTER 3

ATGL driven autophagy/lipophagy mediates hepatic lipid droplet catabolism and fatty acid efflux

Aishwarya Sathyanarayan, Mara T. Mashek, Dana Yao, Chi Chen, Christina Leopold, Nemanja Vujic, Dagmar Kratky and Douglas G. Mashek

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SUMMARY

Hepatic lipid droplet (LD) catabolism is thought to occur via cytosolic lipases such as adipose triglyceride lipase (ATGL) or through autophagy of LDs, a process known as lipophagy. We tested the potential interplay between these metabolic processes and its effects on hepatic lipid metabolism. We showed that hepatic ATGL was both necessary and sufficient to induce autophagy and lipophagy. Following previous work showing that ATGL promotes sirtuin 1 (SIRT1) activity, studies in liver-specific *SIRT1*$^{-/-}$ mice and in primary hepatocytes revealed that SIRT1 was required for ATGL-mediated induction of autophagy and lipophagy. Moreover, lipophagy was required for ATGL to promote LD catabolism. ATGL promoted lysosomal-mediated efflux of FAs from hepatocytes, which occurs prior to their reuptake and utilization in β-oxidation or reesterification pathways. Taken together, these studies show that ATGL-mediated signaling promotes autophagy/lipophagy to control hepatic LD catabolism and FA efflux.

INTRODUCTION

The LD is the major storage organelle of intracellular triacylglycerol (TAG) and, therefore, is the defining characteristic of non-alcoholic fatty liver disease. Historically thought to be inert energy storage depots, LDs are increasingly recognized as dynamic organelles involved in many cellular processes beyond energy storage (Greenberg et al., 2011). Despite significant advances into our understanding of LD catabolism in adipose tissue, there is relatively limited knowledge on the regulation and role of hepatic lipolysis. Studies conducted by our laboratory and others have identified ATGL as a major hepatic lipase that regulates TAG turnover. Specifically, shRNA knockdown or genetic ablation of hepatic *ATGL* promotes steatosis (Ong et al., 2011; Wu et al., 2012), whereas overexpression of *ATGL* in the liver alleviates steatosis (Turpin et al., 2011). Numerous studies show that ATGL specifically channels hydrolyzed fatty acids (FAs) to oxidative pathways and does not influence VLDL secretion (Ong et al., 2011; Wu et al., 2012). Additionally, hepatic ATGL promotes SIRT1 activity to control PPAR-α/PGC-1α signaling and oxidative metabolism (Khan, et al., 2015). Thus, in addition to its prominent roles in tissues such as adipose and heart, ATGL also appears to play a critical role in regulating hepatic energy metabolism and signaling.

Autophagy is a cellular recycling mechanism that provides cells with a source of energy during starvation (He and Klionsky, 2009). Autophagy can be classified into three categories based on lysosomal cargo delivery:

macroautophagy (He and Klionsky, 2009), chaperone-mediated autophagy (Cuervo and Wong, 2014) and microautophagy (Santambrogio and Cuervo, 2011). The role of autophagy in TAG hydrolysis was unclear until Singh et al. showed that inhibiting autophagy promoted LD accumulation and attenuated oxidation of the hydrolyzed FAs (Singh et al., 2009). Since these findings of autophagy-mediated lipid mobilization, termed lipophagy, a plethora of studies have shown the existence of lipophagy in diverse cell types such as macrophages (Ouimet et al., 2011), neurons (Kaushik et al., 2011) and lymphocytes (Hubbard et al., 2010). Less is known in the liver, although recent studies have provided some mechanistic insights into lipophagy. Schroeder et al. identified the small GTPase Rab7 as a key regulator of lipophagy to control hepatic LD degradation (Schroeder et al., 2015). Chaperone-mediated autophagy has also been shown to contribute to hepatic LD catabolism via its degradation of the LD proteins Perilipin 2 and Perilipin 3 to increase association between ATGL and LDs and enhance lipolysis (Kaushik and Cuervo, 2015). Given the above information, we decided to further investigate the link between hepatic ATGL-mediated TAG hydrolysis and autophagy/lipophagy. Herein, we demonstrate that hepatic ATGL promotes LD catabolism and FA oxidation via SIRT1-mediated induction of lipophagy and FA efflux.

EXPERIMENTAL PROCEDURES

Animals, diets and adenovirus administration

All animal protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee or the Austrian Federal Ministry of Science and Research, Division of Genetic Engineering and Animal Experiments, Vienna, Austria. Eight to ten week old C57/Bl6 male mice were housed and acclimatized as previously described (Ong et al. 2011). Adenoviruses to manipulate ATGL and SIRT1 expression were provided by Andrew Greenberg and X.C. Dong respectively, and administered via the tail vein as previously described (Khan, et al., 2015). All mice had free access to water and were fed a purified control diet (TD 94045; Harlan Teklad Premier Laboratory), after adenoviral administration. One week following adenovirus injection, the mice were sacrificed for liver tissue and serum collection after a 16 hour overnight fast (shATGL treatment) or a 4 hour fast (Ad-ATGL treatment). Homozygous SIRT1 floxed mice were purchased from JAX Labs and were cross-bred to Alb-*Cre* transgenic mice to generate liver specific *SIRT1*$^{-/-}$ mice. Wild type and *LIPA*$^{-/-}$ mice (Du et al., 2001a) were maintained in a clean environment with unlimited access to chow diet (4% fat and 19% protein; Altromin 1324, Lage, Germany) and water in a regular light-dark cycle (12 hour light, 12 hour dark).

RNA isolation, quantitative real-time PCR analysis

RNA was extracted with Trizol from liver tissues followed by reverse-transcription with SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) to generate cDNA. Gene expression was quantified as described previously (Khan, et al., 2015).

Cell culture, adenoviral infection and radiolabeling

Hepatocytes were isolated as described previously (Ong et al. 2011) and were transduced with adenovirus expressing ATGL or a control null vector virus 4 hours after plating. Experiments measuring lipid incorporation (pulse) to measure TAG turnover or media FA efflux were performed in M199 media (Invitrogen) containing 26 mmol/L sodium bicarbonate, 23 mmol/L HEPES, 50 IU/ml penicillin, 50 µg/ml streptomycin, 100 nmol/L dexamethasone, 11 mmol/L glucose and 100 nmol/L insulin. The following morning the cells were pulsed with 500 µM [1-^{14}C]oleate added to the above media for 2 hours. Some cells were harvested at the end of the pulse period to measure radiolabel incorporation into cellular lipid fractions. The remainder cells were washed with PBS and media were replaced with fresh complete M199 (as described above) lacking insulin for an additional 6-8 hours (chase period) followed by collection of media and cells for lipid extraction. FAs oxidized or effluxed during the chase period are expressed as a percentage of the pulse [^{14}C]TAG. All experiments to determine autophagy induction in hepatocytes were performed in complete M199 without hormones unless specified otherwise. Media FAs were extracted using the Dole

extraction method using Dole reagent, hexane and water in a (5:3:2) ratio (Dole 1956). Lipid samples were separated into different fractions by thin layer chromatography (TLC) and analyzed as described previously (Ong et al., 2014). Transwell studies were performed using a transwell insert with a microporous membrane (Corning™ Costar™ Transwell™ Permeable Supports). “Donor” cells were plated in the transwell (apical portion) and transduced with the specified adenoviruses, followed by an overnight pulse with C-12 BODIPY FA 558/68 (Invitrogen). The following day transwells containing the donor cells were transferred onto wells containing “acceptor” cells plated in the basal portion and FA efflux was monitored after a 4 hour chase period.

Western Blotting

Protein extraction and immunoblotting were performed as described previously (Khan, et al., 2015). LAMP1 and LIPA antibodies were obtained from ABCAM (Cat. No’s: ab24170 and ab36597 respectively). LC3 and ATG5 antibodies were purchased from Novus Biologicals (Cat. No’s: NB100-2220 and NB110-53818 respectively). SIRT1, ATGL and p62 antibodies were purchased from Cell Signaling Technology (Cat. No’s: 8469S, 2439S and 5114S respectively). Beta-Actin antibody was obtained from Bio-Rad (Cat. No: MCA5775GA).

Chemical reagents/Vectors

Lipase inhibitor ATGListatin was purchased from Xcess Biosciences (Cat. No: MC0150-2s) and used in cells at 30 μM concentration in DMSO for 2 hours

(for imaging studies) and up to 8 hours for pulse-chase experiments. Chloroquine was purchased from Sigma-Aldrich (Cat. No: c6628) and Vacuolin1 from Santa Cruz (Cat. No: 351985-151). CB16.2 and LAListat were kind donations from Dr. Concetta DiRusso (University of Nebraksa) and Dr. Paul Helquist (Notre Dame University) respectively. All autophagy inhibitors were used at the specified concentrations for the duration of the chase. The dual reporter plasmid RFP-GFP-LC3 was a kind donation from Dr. Mark McNiven (Mayo Clinic) and was transfected at 250 ng/well. siATG5 was custom synthesized from Qiagen and siLIPA was procured from Sigma-Aldrich (Cat. No: EMU075891). All vectors were administered using Qiagen's Effectene Transfection Reagent.

Confocal imaging

For immunofluorescence microscopy, cells were grown on coverslips and fixed with 4% paraformaldehyde for 30 minutes and blocked with 1% BSA, 10% donkey serum in phosphate buffer saline (PBS). Incubation with primary (overnight at 4°C) and secondary (1 hour at RT) antibodies was done in the above-mentioned blocking buffer. The secondary antibody conjugated to Alexa Fluor 488 or Fluor 567 was purchased from Invitrogen. For LD staining, cells were incubated with LipidTOX™ Deep Red or Green Neutral Lipid Stain (1 μM; Invitrogen) for 30 minutes at 37°C after fixation. For lysosomal staining, cells were incubated with 20 nM Lysotracker Red DND-99 (Invitrogen) for 30 minutes at 37°C before fixation. Nuclei were stained with DAPI (4′, 6-diamidino-2-phenylindole) for 10 minutes followed by mounting onto slides for visualization.

All images were acquired with a Nikon Olympus 200 fluorescence microscope (Carl Zeiss Microscopy) with a 60X oil objective and 0.6 numerical aperture, and prepared using ImageJ (NIH). Images from 5 different fields per well were captured, and experiments were performed in triplicate.

For imaging tissues, paraffin blocks were generated from formaldehyde fixed livers. Slides from these blocks were de-paraffinized using xylene and ethanol washes alternatively. This was followed by an antigen retrieval step which included boiling the slides in 10 mM sodium citrate buffer with 0.5% Tween-20 and subsequently permeabilization in a buffer containing 0.2% Triton X-100 in PBS. Finally the slides were blocked using 10% donkey serum in 1% BSA for 1 hour at room temperature followed by overnight incubation with primary antibody at a dilution of 1:100. The following day, slides were washed and incubated with fluorophore-conjugated secondary antibodies at 1:100 dilution for 1 hour at room temperature, followed by addition of DAPI and subsequent mounting with coverslips.

LC-MS analysis of hepatic lipids

Hepatic lipids were extracted from the liver based on previous methods (Bligh and Dyer, 1959). Liver samples (100 mg) were homogenized in 0.5 ml methanol and mixed with 0.5 ml chloroform and 0.4 ml water. Phase separation was achieved by 10 minute centrifugation at 18,000 g. The lipid fraction in the chloroform phase was dried under nitrogen and then reconstituted in 0.5 ml n-butanol. A 5 µL aliquot of diluted serum or liver lipid sample was injected into an

Acquity^{TM} UPLC system (Waters, Milford, MA) and separated by a gradient of mobile phase ranging from water to 95% aqueous acetonitrile containing 0.1% formic acid over a 10-min run. LC eluents were introduced into a Xevo-G2-S mass spectrometer (Waters) for accurate mass measurement and ion counting. Capillary voltage and cone voltage for electrospray ionization was maintained at 3 kV and 30 V for positive-mode detection, or at -3 kV and -35 V for negative-mode detection, respectively. Source temperature and desolvation temperature were set at 120 °C and 350 °C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (600 L/h), and argon as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range m/z 50-1000) and monitored by the intermittent injection of the lock mass leucine enkephalin ($[M+H]^+$ = 556.2771 m/z and ($[M+H]^-$ = 554.2615 m/z) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynxTM software (Waters) in centroided format. Additional structural information was obtained tandem MS fragmentation with collision energies ranging from 15 to 30 eV.

Statistical Analysis

Statistical comparisons were made using analysis of variance or student t-test. All data are presented as means ± SEM and statistical significance was declared at $P<0.05$.

RESULTS

Hepatic ATGL regulates autophagy/lipophagy

We have previously shown that hepatic ATGL plays an important role in TAG turnover and the subsequent channeling of hydrolyzed FAs to oxidative pathways as well as upregulating PPAR-α/PGC-1α signaling to control oxidative metabolism (Ong et al., 2011). Recent evidence indicates that ATGL exhibits multiple LC3-interacting region motifs (LIRs), which are required for LD catabolism (Martinez-Lopez et al., 2016). Given that lipophagy also contributes to hepatic LD catabolism (Schroeder et al., 2015; Settembre et al., 2013; Singh et al., 2009), we sought to dissect the links between hepatic ATGL-catalyzed lipolysis and autophagy/lipophagy. Inhibition of ATGL via shRNA-mediated knockdown, as previously reported (Ong et al., 2014), or chemical inhibition via ATGListatin in liver or primary hepatocytes, respectively, significantly lowered the expression of autophagy genes (Figures 1A-B). *ATGL* knockdown decreased hepatic protein levels of LC3II and LAMP1, but increased p62 when compared to the scrambled controls (Figures 1C and 7A) consistent with reduced autophagy. ATGListatin also reduced LC3 punctae formation in starved MEFs (Figures 1D and 7B). To measure autophagic flux, we used the tandem GFP-RFP-LC3 sensor (Kimura et al. 2007), which contains an acid-labile GFP and acid-resistant RFP to distinguish between autophagosome and autolysosome localization. We observed increased RFP signal relative to GFP in the controls, which was abrogated in response to chemical inhibition of ATGL indicating, reduced

autophagic flux (Figure 1E). ATGL knockdown in mouse livers also reduced LC3 staining and its colocalization with LDs indicating reduced macrolipophagy (Figure 1F). Similarly, ATGListatin reduced localization of lysosomes with LDs in primary hepatocytes suggesting suppressed microlipophagy (Figures 1G and 7C).

In contrast to ATGL knockdown or inhibition, the livers of Ad-*ATGL* treated mice described in (Ong et al., 2011) had increased expression of autophagy genes along with elevated LAMP1 and LC3II and reduced p62 protein levels (Figures 2A-B and Figure 7D). ATGL overexpression increased colocalization of LDs with LC3 and lysosomes suggesting increased macro- and microlipophagy (Figures 2C-D). ATGL overexpression also enhanced autophagic flux in hepatocytes transfected with the dual sensor plasmid compared to the control (Figure 2E). Thus, these results show that ATGL is a positive regulator of both autophagy and lipophagy in the liver.

SIRT1 mediates the effects of ATGL on autophagy induction

We have shown that hepatic ATGL regulates mitochondrial biogenesis and the expression of genes involved in β-oxidation through its regulation of SIRT1 (Khan, et al., 2015). Additionally, SIRT1 is a well-established activator of autophagy through its deacetylation and activation of key components of the autophagy induction network such as the ATG protein family (Lee et al., 2008) and transcriptional regulators including PGC-1α, FoxO1, CREB, PPAR-α,

ChREBP and FXR (Brunet et al., 2004; Kemper et al., 2009; Qiang et al., 2011; Rodgers et al., 2005; Wang et al., 2010). An alternate transcription factor TFEB has also been shown to control cellular lipid metabolism in a starvation-autophagy dependent manner (Settembre et al., 2013) and downstream regulation of the transcriptional levels of LAMP1 and lysosomal exocytosis (Medina et al., 2011). To examine the importance of SIRT1 in mediating the effects of ATGL on hepatic autophagy, we transduced liver-specific *SIRT1*$^{-/-}$ mice (L-*SIRT1*$^{-/-}$) with an adenovirus harboring *ATGL*. Consistent with data from Figure 2A, Ad-*ATGL* increased gene expression of several autophagy targets, but this effect was abolished in the absence of *SIRT1* (Figure 3A). Western blot analysis revealed increased expression of LAMP1, ATG5 and LC3II with *ATGL* overexpression in L-*SIRT*$^{flox/flox}$ mice, but this effect was completely lost in the absence of hepatic *SIRT1* (Figure 3B). To support our findings from the L-*SIRT1*$^{-/-}$ mouse data, we also used a dual adenovirus system to simultaneously overexpress *ATGL* and knockdown *SIRT1* (shSIRT1) in the liver (Khan, et al., 2015) to more acutely regulate SIRT1. Ad-*ATGL* treated livers had increased expression of autophagy genes, which was completely abolished when *SIRT1* was knocked down (Figure 8A). Similarly, *ATGL* overexpression increased LAMP1 protein levels, which required SIRT1 (Figure 8B). In addition, *SIRT1* ablation abrogated the induction of TAG turnover and oxidation of hydrolyzed FA oxidation in response to ATGL expression in primary hepatocytes (Figures 3C-D and Figure 8C). L-*SIRT1*$^{-/-}$ mice also had lower ketone body production

compared to the floxed controls in response to ATGL expression (Figure 3E). Ad-*ATGL* enhanced LC3 colocalization with LDs in primary hepatocytes, which was reduced when cells were treated with the SIRT1 inhibitor EX527 (Figure 3F). Similar results were observed in the interaction between lysosomes and LDs (Figures 3G and 8D). Finally, the increase in autophagic flux in response to Ad-*ATGL* was negated by EX527 treatment (Figure 8E). Taken together, these data show that SIRT1 mediates the effects of ATGL on promoting autophagy/lipophagy.

Lipophagy mediates the effects of ATGL on TAG catabolism and FA oxidation

Since ATGL promotes lipophagy, we next explored if lipophagy was responsible for mediating the effects of ATGL on TAG catabolism and the subsequent oxidation of hydrolyzed FAs. Consistent with previous studies (Ong et al., 2011; Sapiro et al., 2009), overexpressing *ATGL* decreased TAG stores, but this effect was partially or completely abrogated when autophagy was blunted via exposure of hepatocytes to the autophagy inhibitor chloroquine or knockdown of ATG5 (Figures 4A-B). Moreover, blocking lipophagy specifically, via the lysosomal acid lipase (LIPA) inhibitor Lalistat (Pearson et al., 2014), prevented the increase in TAG turnover in response to *ATGL* overexpression (Figure 4C). We have previously shown that ATGL channels hydrolyzed FAs to the mitochondria for β-oxidation (Ong et al., 2011), thus, we further tested if autophagy/lipophagy was required for this effect. Similar to the TAG turnover

data, blocking autophagy or lipophagy attenuated the oxidation of hydrolyzed FAs following *ATG*L overexpression (Figures 4D-F). Consistent with the above results, over-expressing *ATGL* reduced total lipid stores in hepatocytes and this effect was abrogated when autophagy/lipophagy was blocked (Figure 4G). Finally, we analyzed livers from whole body *LIPA*$^{-/-}$ mice that were injected with control or *ATGL* expression adenoviruses. ATGL overexpression was confirmed through enhanced protein expression (Figure 9A) and increased TAG hydrolase activity (Figure 9B). Histological analysis revealed pronounced hepatomegaly, steatosis and foam cell nests in *LIPA*$^{-/-}$ mice (Figure 4H) as previously reported (Du et al., 2001b), however, *ATGL* overexpression did not influence liver histology and was unable to reduce TAG stores or increase serum ketone bodies, as previously seen in wild type mice (Ong et al., 2014; Turpin et al., 2011), in the absence of *LIPA* (Figures 4I-K). Thus, these data show that lipophagy mediates the effects of ATGL on LD catabolism and the subsequent oxidation of liberated FAs.

Hepatic ATGL promotes FA efflux from cells via the lysosome

We have shown that FA binding proteins including L-FABP, the major liver FABP, are not required for the effects of ATGL on oxidation of hydrolyzed FAs (Ong et al., 2014), suggesting that an alternate route exists to transport hydrolyzed FAs to the mitochondria for oxidation. Since lysosomes can fuse with the plasma membrane to release their luminal contents extracellularly (Samie

and Xu, 2014), we next explored if FAs generated from lipophagy were effluxed. The addition of 2% BSA to the chase media to bind and sequester FAs resulted in a robust increase in FA efflux, and the increase in FA efflux in *ATGL* overexpressing cells required the presence of BSA (Figure 5A). Similarly, *ATGL* knockdown reduced FA efflux by almost 90% (Figure 5B). Transwell studies revealed that *ATGL* overexpression in donor hepatocytes increased the transfer and accumulation of C12 BODIPY in acceptor hepatocytes (Figure 5C). Since BSA only binds FAs to slow, but not prevent, their uptake, we used a chemical inhibitor of FA uptake, CB16.2, (Sandoval et al., 2010) as an additional approach to measure efflux. Addition of CB16.2 during the chase period resulted in a nearly 15-fold increase in FA efflux in control hepatocytes and further increased extracellular FAs in response to *ATGL* overexpression (Figure 5D). Next, we investigated if ATGL-mediated FA efflux was lipophagy-dependent. The addition of Lalistat or chloroquine or knockdown of LIPA (Figure 9C) significantly attenuated FA efflux in response to ATGL overexpression (Figures 5E-G). Adding an alternate lysosomal turnover inhibitor, Vacuolin1, which prevents the Ca^{2+}-dependent fusion of the lysosomes to the cell membrane (Cerny et al., 2004), blocked the effects of *ATGL* overexpression on FA efflux (Figure 5H). Addition of Vacuolin 1 in transwell studies prevented the transfer and accumulation of FAs in acceptor cells co-cultured with hepatocytes overexpressing *ATGL* (Figure 9D). Vacuolin 1 caused ballooning of lysosomes and nearly all LDs were found within lysosomes (Figures 5I) or interacting with

lysosomes (Figure 9E), thus, highlighting the importance of lysosomal turnover in LD catabolism. Addition of a FABP inhibitor, HTS01307, showed no difference in effluxed FAs, thus, further suggesting that cytosolic shuttling of hydrolyzed FAs does not contribute to FA efflux (Figure 9F).

We proceeded to characterize FA efflux using *in situ* perfused livers from mice fed or fasted for 16 hours. Under all conditions tested, overnight fasted mice had increased FA efflux compared to fed mice (Figure 5J). As observed in the *in vitro* models, FA efflux was increased by the addition of BSA and further increased in the presence of CB16.2. Finally, addition of vacuolin 1 significantly blocked FAs released from the liver.

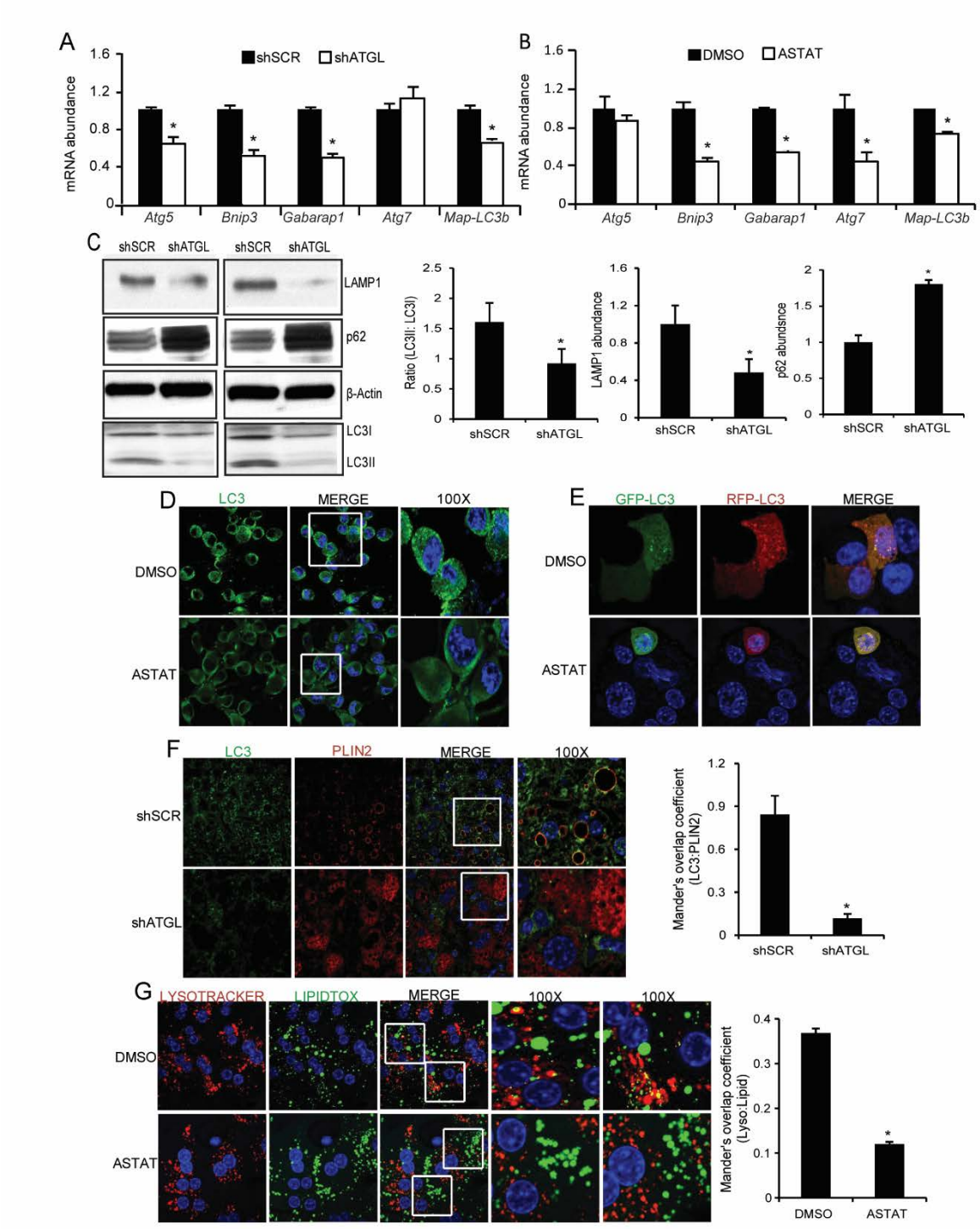
Next we performed lipidomic analysis on liver samples isolated from control and sh*ATGL* adenovirus administered mice. Significantly higher levels of sphingomyelin species were found in samples from mice treated with *ATGL* shRNA (Figures 10A-B). Sphingomyelins are converted to ceramides by both lysosomal and Golgi-specific sphingomyelinases (*SMPD1*/*SMPD2*) (Kolesnick, 2002). Correspondingly, the gene expression of *SMPD1* and *SMPD2* was significantly lower in the sh*ATGL* samples compared to the control (Figure 10C). Sphingomyelins are known to be negative regulators of lysosomal Ca^{2+} channel protein transient receptor potential cation channel mucolipin, subfamily member 1 (TRPML1) (Shen et al., 2012). TRPML1 channels are involved in lysosomal trafficking and expulsion of lysosomal contents, and its dysfunction results in several lysosomal storage disorders such as Niemann-Pick Disease Type A and

C (NP-A and C) (Lloyd-Evans and Platt, 2011; Soyombo et al., 2006; Wang et al., 2015). We used a TRPML1 agonist (MLSA1) to induce lysosomal Ca^{2+} leak and found more effluxed FAs (Figure 10D). Concomitantly, cells treated with sphingomyelins had lower FA efflux compared to the controls. Taken together, these data indicate that ATGL promotes FA efflux via lysosome fusion to the plasma membrane.

ATGL-mediated FA efflux requires reuptake to be channeled towards oxidation or TAG synthesis

FAs liberated from TAG stored in LDs are thought to be either reesterfied or used in downstream metabolic pathways including FA oxidation. Our findings indicate that hydrolyzed FAs are effluxed and undergo reuptake prior to further metabolism. To further interrogate this potential pathway, we quantified the metabolism of FAs in response to manipulations of FA efflux. As expected, using Vacuolin 1 to inhibit FA efflux significantly attenuated the loss of [^{14}C]TAG and the oxidation of hydrolyzed FAs in response to *ATGL* overexpression (Figures 6A-B). The addition of BSA in the chase media to sequester expelled FAs was sufficient to lower cellular [^{14}C]TAG levels in both Ad-Null and Ad-*ATGL* treated hepatocytes and attenuated the increase in oxidation in cells overexpressing ATGL (Figures 6C-D). Similarly, inhibition of FA uptake with CB16.2 lowered cellular [^{14}C]TAG in both treatment groups and blocked the induction of FA oxidation in *ATGL* overexpressing cells (Figures 6E-F). We next labeled cells

with [^{3}H]glycerol during the chase period to directly determine if rates of TAG synthesis were altered in response to BSA and CB16.2. Ad-*ATGL* increased the [^{3}H]glycerol incorporation into TAG consistent with more FAs being available as substrates for *de novo* TAG synthesis (Figure 6G). However, BSA alone or in addition to CB16.2 reduced *de novo* TAG synthesis and abrogated the increase in TAG synthesis in response to ATGL overexpression. Additionally, ATGListatin or LAListat had minor effects on reducing FA oxidation in cells treated with CB16.2 further highlighting the significance of efflux prior to oxidation (Figure 6H). Finally, we measured β-hydroxybutyrate in the liver perfusates of the fed and fasted mice treated with BSA, CB16.2 and vacuolin 1 described in Figure 5J. Although β-hydroxybutyrate did not reach statistical significance in the fasted mice, sequestering effluxed FAs (BSA and CB16.2) or preventing lysosome-mediated efflux (vacuolin 1) significantly reduced hepatic FA oxidation (Figure 6I). Taken together, these data strongly support a model where ATGL promotes FA efflux, which occurs prior to FA reuptake and subsequent oxidation or resterification in TAG.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Bnip3
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MERGE
100X
100X
DMSO
ASTAT
Mander's overlap coefficient (Lyso:Lipid)
DMSO
ASTAT

Figure 1. Liver-specific inhibition of ATGL attenuates autophagy/lipophagy.

(A) *In vivo* ablation of hepatic ATGL via an *ATGL* shRNA adenovirus reduced the expression of autophagy genes (n=5); *P<0.05 vs. control shRNA. (B) Inhibition of ATGL in primary hepatocytes with ATGListatin (Astat, 30μM for 36 hours) decreased autophagy gene expression (n=5); *P<0.05 vs. DMSO. (C) In vivo ATGL knockdown reduces the LC3II/LC3I ratio and protein levels of LAMP1, and increased p62 expression; a representative western blot and densitometry from 3 mice is shown. (D) Astat decreased LC3 puncta in mouse embryonic fibroblasts (MEFs) (n=3). € Hepatocytes transfected with the dual RFP-GFP-LC3 plasmid were treated with Astat to acutely inhibit ATGL. Increased red punctae is indicative of enhanced lysosomal activity as observed with the DMSO treatment when compared to the Astat treated cells (n=4). (F) Confocal imaging of liver sections show reduced LD localizatiob (as measured with the LD protein perilipin 2, PLIN2) with LC3 in response to ATGL knockdown. (G) Astat decreased lysosomal association with LDs in primary mouse hepatocytes (n=3 for F and G).

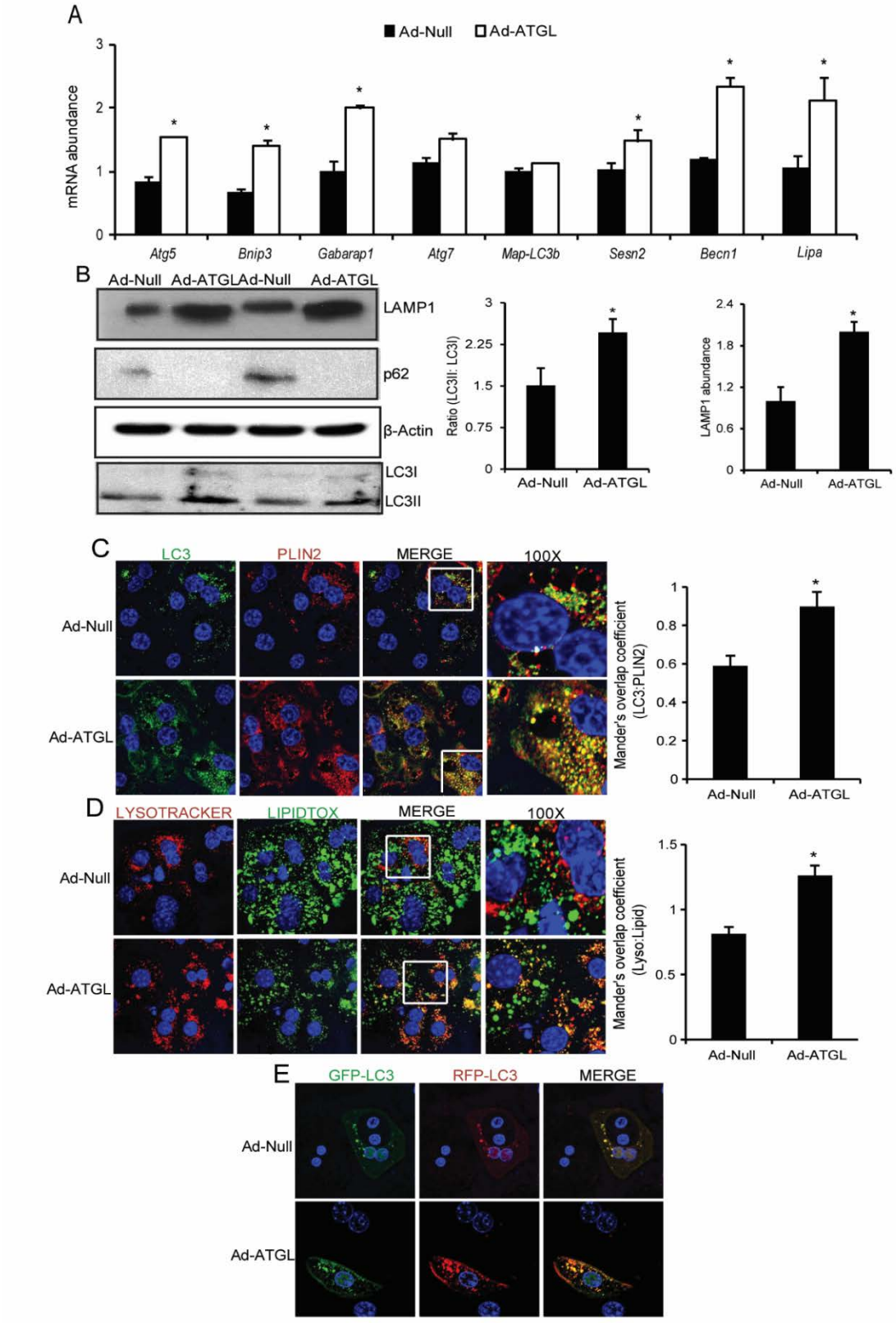
A
Ad-Null
Ad-ATGL
mRNA abundance
Atg5
Bnip3
Gabarap1
Atg7
Map-LC3b
Sesn2
Becn1
Lipa
B
Ad-Null
Ad-ATGL
Ad-Null
Ad-ATGL
LAMP1
p62
β-Actin
LC3I
LC3II
Ratio (LC3II: LC3I)
LAMP1 abundance
C
LC3
PLIN2
MERGE
100X
Ad-Null
Ad-ATGL
Mander's overlap coefficient (LC3:PLIN2)
D
LYSOTRACKER
LIPIDTOX
MERGE
100X
Mander's overlap coefficient (Lyso:Lipid)
E
GFP-LC3
RFP-LC3
MERGE

Figure 2. ATGL overexpression is sufficient to promote autophagy/lipophagy.

(A) *In vivo* overexpression of hepatic ATGL increased autophagy target gene expression (n=5); *P<0.05 vs. Ad-Null. (B) Hepatic ATGL overexpression increased the LC3II/LC3I ratio and LAMP1 protein levels as analyzed via western blotting. Livers overexpressing ATGL had no visible bands for p62 indicative of enhanced autophagosome clearance; a representative Western blot from 3 mice is shown. (C-D) *In vivo* overexpression of hepatic ATGL increased LC3 and lysosomal co-localization with LDs stained with PLIN2 or lipidtox. (E) The dual reporter RFP-GFP-LC3 was transfected in primary hepatocytes that were transduced with Ad-Null or Ad-*ATGL* adenoviruses; (n=3 for C-E).

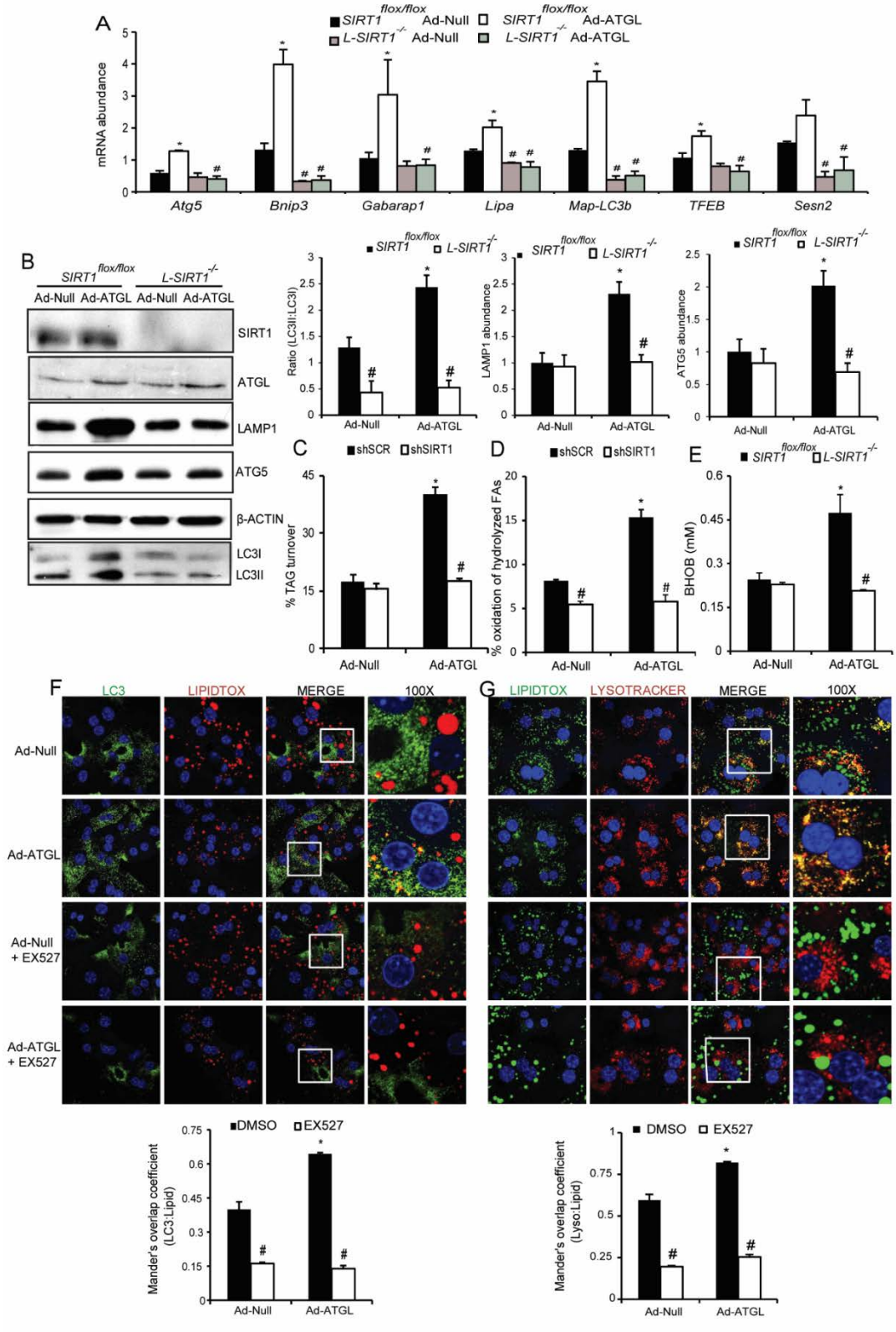
A
SIRT1 flox/flox Ad-Null
SIRT1 flox/flox Ad-ATGL
L-SIRT1 -/- Ad-Null
L-SIRT1 -/- Ad-ATGL
mRNA abundance
Atg5
Bnip3
Gabarap1
Lipa
Map-LC3b
TFEB
Sesn2
B
SIRT1 flox/flox
L-SIRT1 -/-
Ad-Null Ad-ATGL Ad-Null Ad-ATGL
SIRT1
ATGL
LAMP1
ATG5
β-ACTIN
LC3I
LC3II
Ratio (LC3II:LC3I)
LAMP1 abundance
ATG5 abundance
C
shSCR shSIRT1
% TAG turnover
D
shSCR shSIRT1
% oxidation of hydrolyzed FAs
E
BHOB (mM)
F
LC3
LIPIDTOX
MERGE
100X
Ad-Null
Ad-ATGL
Ad-Null + EX527
Ad-ATGL + EX527
G
LIPIDTOX
LYSOTRACKER
MERGE
100X
DMSO
EX527
Mander's overlap coefficient (LC3:Lipid)
Mander's overlap coefficient (Lyso:Lipid)

Figure 3. SIRT1 mediates the effects of ATGL on autophagy/lipophagy.

(A) The ATGL-mediated increase in autophagy gene expression was abolished in the absence of SIRT1. *SIRT1*$^{flox/flox}$ or L-*SIRT1*$^{-/-}$ were treated with Ad-Null or Ad-*ATGL* viruses for 7 days prior to sacrifice (n=6); *P<0.05 vs. Ad-Null and $^{\#}$P<0.05 vs. *SIRT1*$^{+/+}$. (B) Protein expression of autophagy genes in response to ATGL overexpression and/or SIRT1 ablation; a representative western blot and densitometry analysis from 3 mice is shown. (C-D) Primary mouse hepatocytes were treated with dual adenoviruses and TAG turnover and FA oxidation during the chase period were measured (n=5); *P<0.05 vs. Ad-Null and $^{\#}$P<0.05 vs. shSCR. (E) L-*SIRT1*$^{-/-}$ mice treated with Ad-ATGL had lower β-hydroxybutyrate compared to the floxed controls (n=6); *P<0.05 vs. Ad-Null and $^{\#}$P<0.05 vs. *SIRT1*$^{+/+}$. (F-G) Confocal imaging revealed that Ad-*ATGL* enhanced LC3-LD and lysosome-LD colocalization, which was lost with 10mM EX527, a SIRt1 inhibitor (n=2).

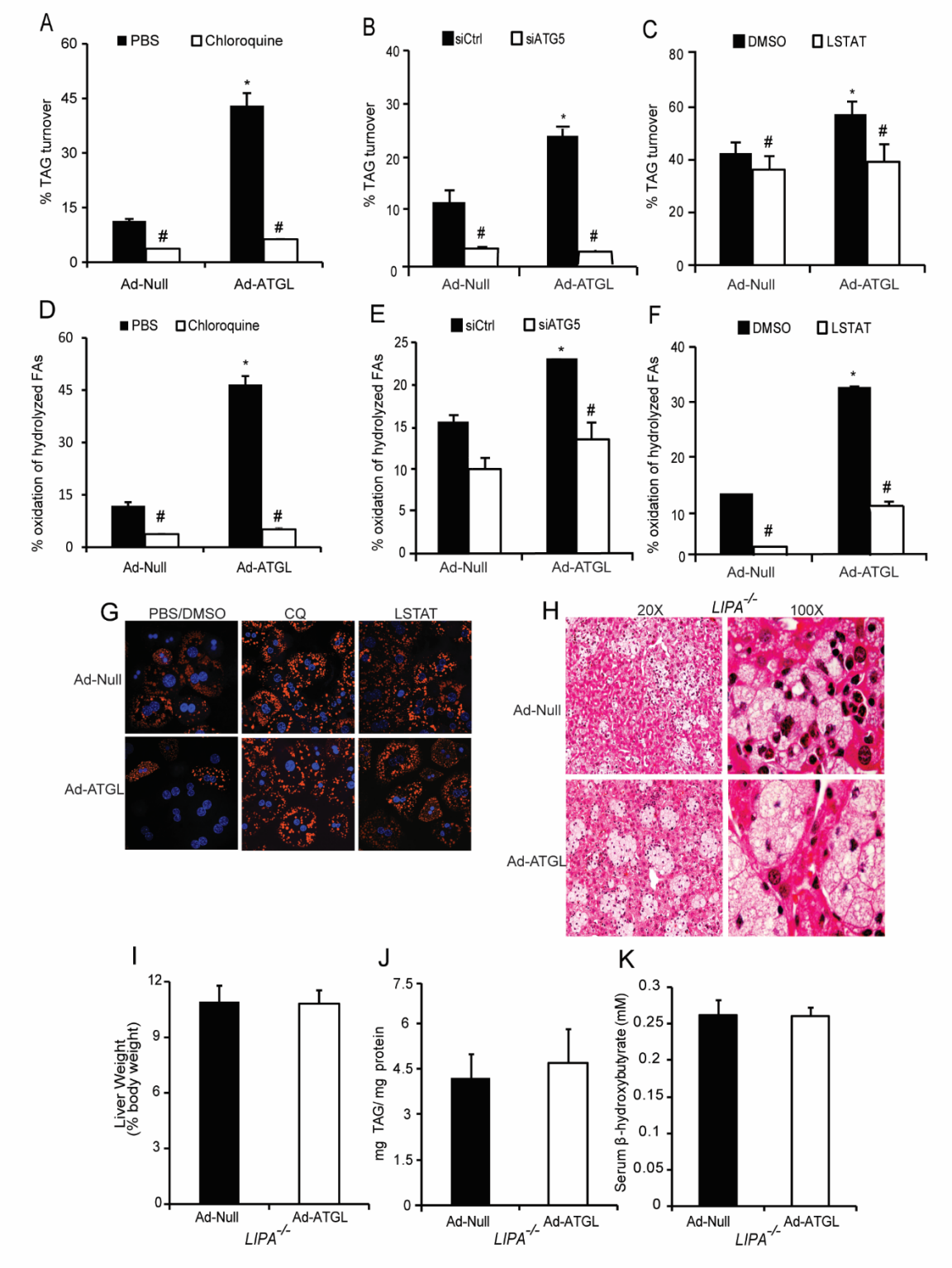
A
PBS
Chloroquine
% TAG turnover
Ad-Null
Ad-ATGL
B
siCtrl
siATG5
% TAG turnover
C
DMSO
LSTAT
% TAG turnover
D
PBS
Chloroquine
% oxidation of hydrolyzed FAs
E
siCtrl
siATG5
% oxidation of hydrolyzed FAs
F
DMSO
LSTAT
% oxidation of hydrolyzed FAs
G
PBS/DMSO
CQ
LSTAT
Ad-Null
Ad-ATGL
H
20X
LIPA-/-
100X
Ad-Null
Ad-ATGL
I
Liver Weight (% body weight)
Ad-Null
Ad-ATGL
LIPA-/-
J
mg TAG/ mg protein
Ad-Null
Ad-ATGL
LIPA-/-
K
Serum β-hydroxybutyrate (mM)
Ad-Null
Ad-ATGL
LIPA-/-

Figure 4. Lipophagy is required for ATGL-mediated effects on TAG catabolism.

Isolated primary mouse hepatocytes were transduced with Ad-Null or Ad-*ATGL* adenoviruses and treated with 600 μM chloroquine (A), siATG5 (B), 10 μM LAListat (C) or their respective controls. Ad-ATGL increased TAG turnover, which was abolished with inhibition of autophagy/lipophagy. (D-F) Autophagy/lipophagy inhibitors impaired the ATGL-mediated induction in FA oxidation (n=6 for A-F); *P<0.05 vs. Ad-Null and $^{\#}$P<0.05 vs. siCtrl or vehicle. (G) Chloroquine and LAListat, added during the chase resulted in increased LD staining and blocked LD depletion in response to ATGL overexpression. Primary mouse hepatocytes were pulsed with a C-12 BODIPY FA (558/68) overnight, followed by an 8 hour chase in insulin free M199 along with inhibitors (n=3). (H) H&E staining of liver sections from *LIPA*$^{-/-}$ treated with Ad-Null or Ad-*ATGL* adenoviruses. ATGL overexpression did not influence liver weights (I), liver TAG (J) or serum ketone bodies (K) in *LIPA*$^{-/-}$ mice (n=6).

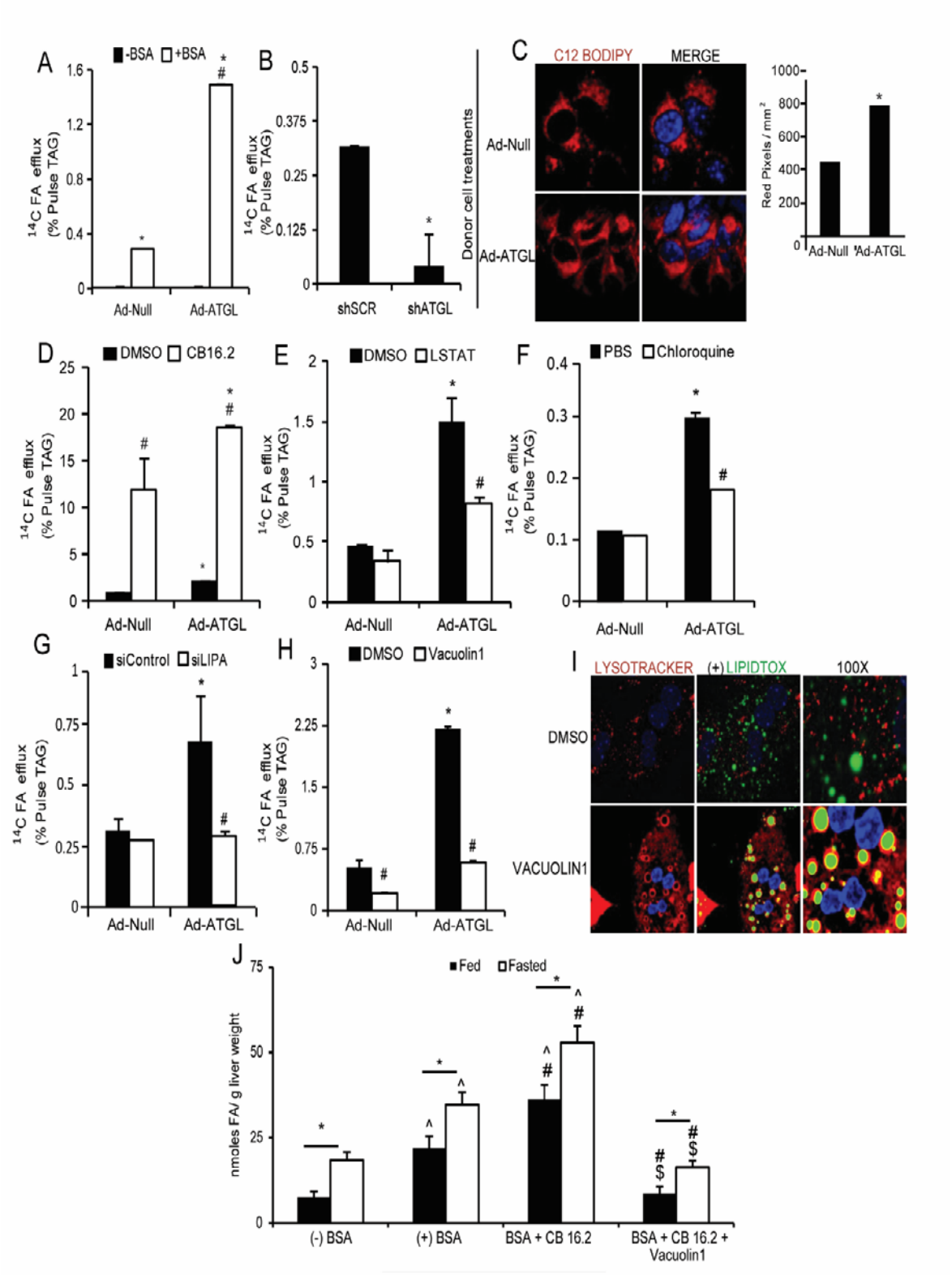
A
-BSA
+BSA
^{14}C FA efflux (% Pulse TAG)
Ad-Null
Ad-ATGL
B
shSCR
shATGL
C
C12 BODIPY
MERGE
Donor cell treatments
Ad-Null
Ad-ATGL
Red Pixels / mm^2
D
DMSO
CB16.2
E
DMSO
LSTAT
F
PBS
Chloroquine
G
siControl
siLIPA
H
DMSO
Vacuolin1
I
LYSOTRACKER
(+) LIPIDTOX
100X
DMSO
VACUOLIN1
J
Fed
Fasted
nmoles FA/ g liver weight
(-) BSA
(+) BSA
BSA + CB 16.2
BSA + CB 16.2 + Vacuolin1

Figure 5. ATGL mediates FA release from cells via the lysosome. (A-B) Pulse-chase experiments show that hepatocytes transduced with Ad-*ATGL* increased and with Ad-sh*ATGL* decreased media FAs in the presence of 2% BSA in the chase media (n=3). *P<0.05 vs. no BSA or shSCR and $^{\#}$P<0.05 vs. Ad-Null. (C) Transwell studies revealed that Ad-*ATGL* transduced donor hepatocytes released more BODIPY FAs to untransduced acceptor hepatocytes compared to the Ad-Null controls (n=3). (D) Inhibiting reuptake of expelled FAs using the FA uptake inhibitor CB16.2 (100 μM) along with BSA increased media FA content under basal and Ad-ATGL conditions (n=4). (E, F, G) The autophagosome-lysosome fusion inhibitor chloroquine, LIPA inhibitor (Lstat) or using a siRNA targeting *LIPA* lowered media FAs (n=3). (H) vacuolin 1 (75 μM), which inhibits lysosomal docking to the cell membrane, decreased media FAs in Ad-Null treated hepatocytes and largely blocked the induction of media FAs in hepatocytes overexpressing ATGL (n=6). (I) Imaging studies revealed a ballooning effect coupled with lipid accumulation within lysosomes 4 hours post vacuolin 1 (75 μM) treatment (n=3). For Figures D-H, *P<0.05 vs. Ad-Null and $^{\#}$P<0.05 vs. vehicle or siControl. (J) *In vivo* efflux of FAs from liver perfusates. Fasting and addition of 1% BSA significantly increased FA sequestration, which was further increased with the addition of 100 μM CB16.2 and negated in the presence of 75 μM vacuolin 1 (n=6). ^P<0.05 vs. -BSA,$^{\#}$P<0.05 vs. +BSA, $^{\$}$P<0.05 vs. BSA+CB16.2.

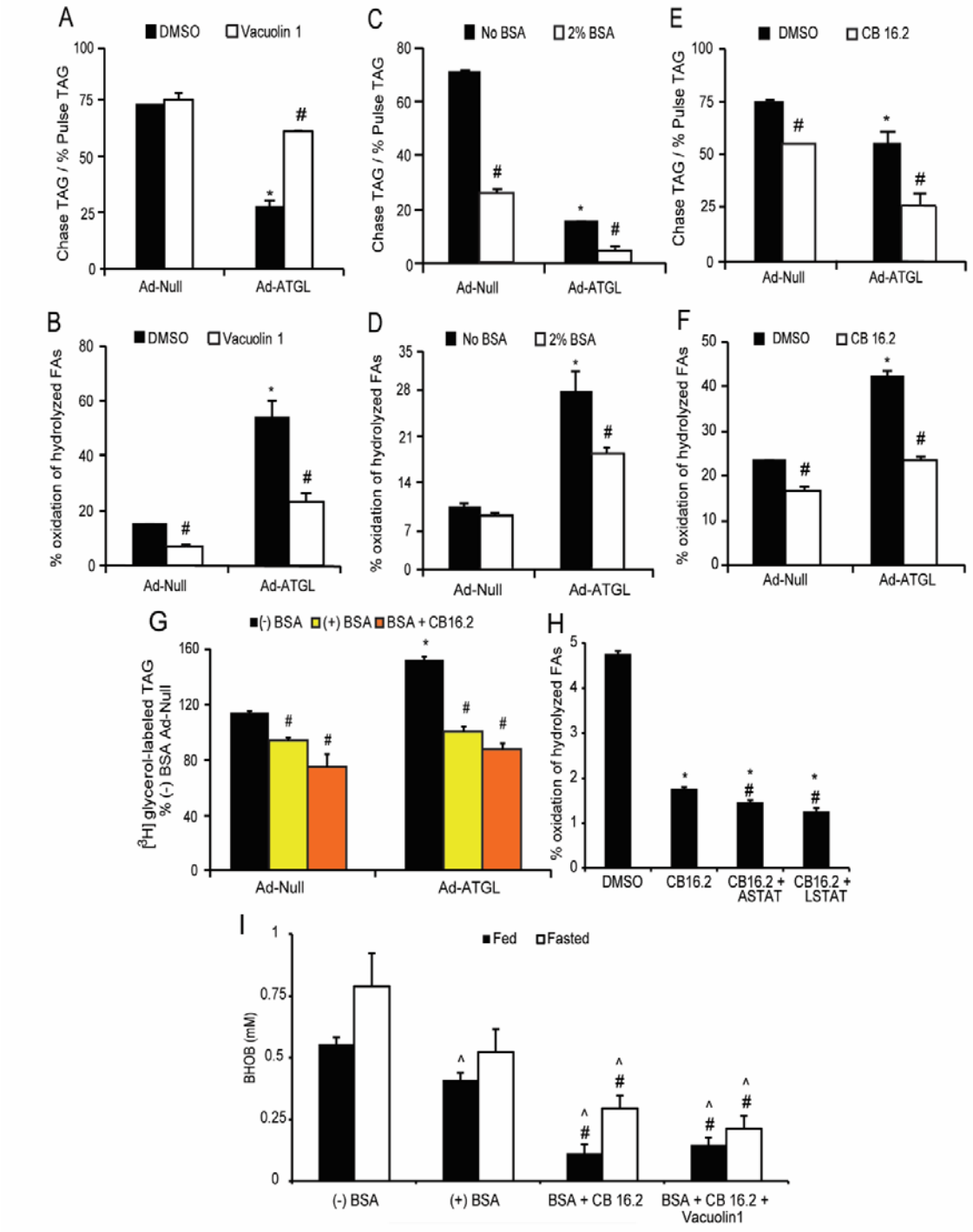
A
DMSO
Vacuolin 1
Chase TAG / % Pulse TAG
Ad-Null
Ad-ATGL
C
No BSA
2% BSA
Chase TAG / % Pulse TAG
Ad-Null
Ad-ATGL
E
DMSO
CB 16.2
Chase TAG / % Pulse TAG
Ad-Null
Ad-ATGL
B
DMSO
Vacuolin 1
% oxidation of hydrolyzed FAs
Ad-Null
Ad-ATGL
D
No BSA
2% BSA
% oxidation of hydrolyzed FAs
Ad-Null
Ad-ATGL
F
DMSO
CB 16.2
% oxidation of hydrolyzed FAs
Ad-Null
Ad-ATGL
G
(-) BSA
(+) BSA
BSA + CB16.2
[3H] glycerol-labeled TAG % (-) BSA Ad-Null
Ad-Null
Ad-ATGL
H
% oxidation of hydrolyzed FAs
DMSO
CB16.2
CB16.2 + ASTAT
CB16.2 + LSTAT
I
Fed
Fasted
BHOB (mM)
(-) BSA
(+) BSA
BSA + CB 16.2
BSA + CB 16.2 + Vacuolin1

Figure 6. Expelled FAs require re-uptake before their subsequent metabolism.

(A-B) vacuolin 1 (75 μM) prevented the loss in cellular TAG and oxidation of hydrolyzed FAs in response to ATGL overexpression (n=6). (C-D) Addition of 2% BSA to the chase and/or ATGL overexpression reduced [^{14}C]TAG during the chase period and BSA attenuated the increase in oxidation of hydrolyzed FAs following ATGL overexpression (n=6). (E-F) Addition of 100 μM of CB16.2 reduced [^{14}C]TAG (E) and blocked the ATGL-mediated increase in oxidation of hydrolyzed FAs during the chase period (F) (n=4). (G) ATGL overexpression increased [^{3}H]glycerol incorporation into cell TAG during the chase period, an effect that was blocked by the addition of 2% BSA alone or with 100 μM CB16.2. Quantification data are from n=3. For A-G, *P<0.05 vs. Ad-Null and $^{\#}$P<0.05 vs. vehicle. (H) Incubation of hepatocytes during a 2-hour chase period with ATGListatin or LAListat in the presence of the FA reuptake inhibitor CB16.2 has a significant but subtle effect on the oxidation of hydrolyzed FAs (n=3). *P<0.05 vs. DMSO and $^{\#}$P<0.05 vs. CB16.2. (I) BSA independently or in combination with CB16.2 and vacuolin 1 lowered β-hydroxybutyrate concentrations in the perfusate (n=6). ^P<0.05 vs. –BSA, $^{\#}$P<0.05 vs. +BSA.

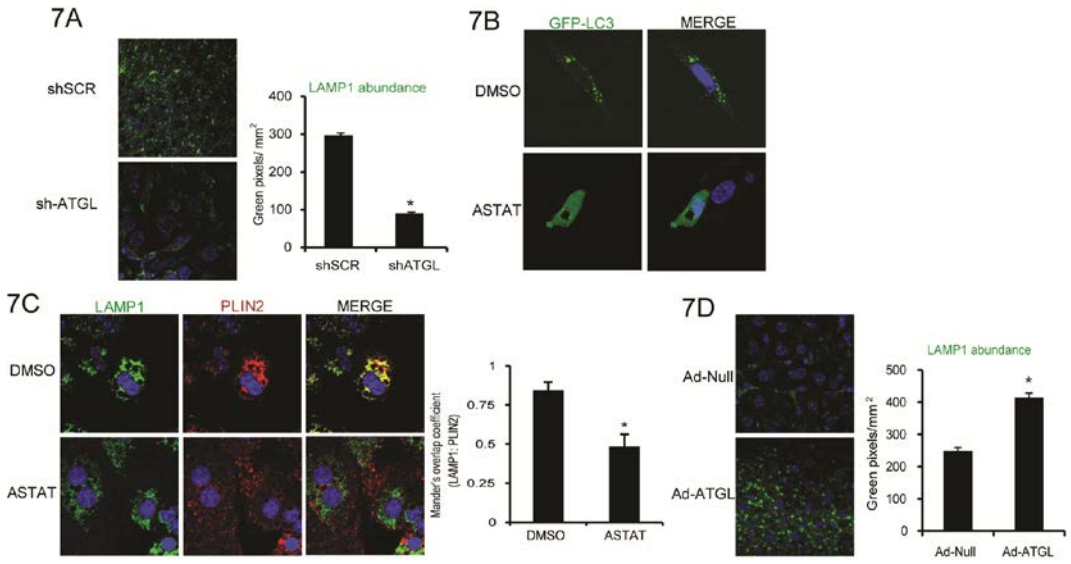

Figure 7. ATGL promotes FA oxidation and autophagy/lipophagy.

(7A) Ad-shATGL treated mice had lower levels of hepatic LAMP1 protein (n=2); *P<0.05. (7B) MEFs were transfected with GFP-LC3 and starved in Earle's Balanced Salt Solution (EBSS) for 2 hours. GFP punctae are visible in the DMSO treated cells and are lost with ATGListatin treatment (n=2); *P<0.05. (7C) Primary hepatocytes cultured in EBSS for 2 hours with DMSO or ATGListatin and stained for lysosomes (LAMP1) and LDs (PLIN2). Colocalization between LAMP1 and PLIN2 was reduced with ATGListatin treatment (n=3). (7D) Ad-ATGL treated mice had increased hepatic LAMP1 protein.

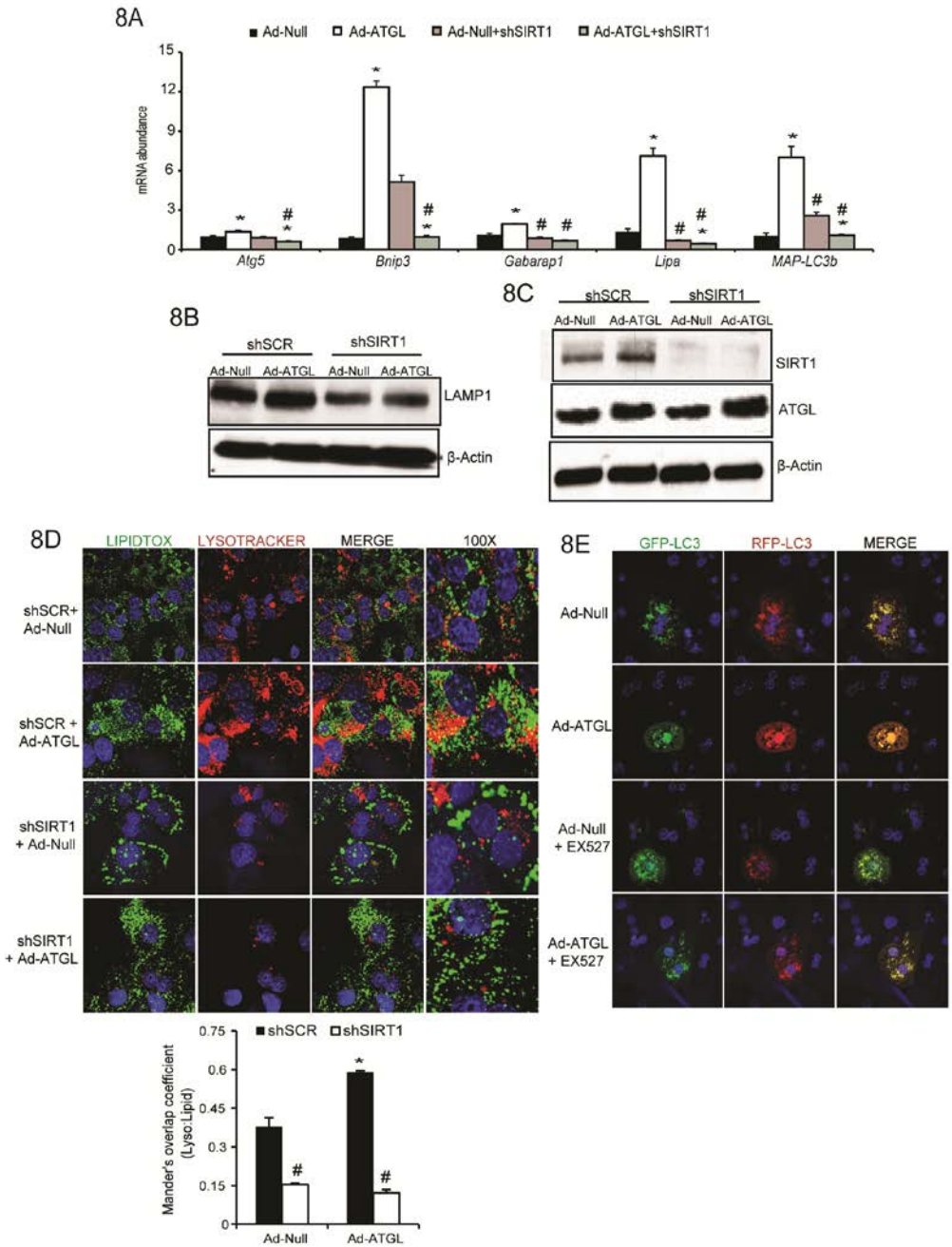
8A
Ad-Null
Ad-ATGL
Ad-Null+shSIRT1
Ad-ATGL+shSIRT1
mRNA abundance
Atg5
Bnip3
Gabarap1
Lipa
MAP-LC3b
8B
shSCR
shSIRT1
Ad-Null
Ad-ATGL
LAMP1
β-Actin
8C
SIRT1
ATGL
8D
LIPIDTOX
LYSOTRACKER
MERGE
100X
shSCR+ Ad-Null
shSCR + Ad-ATGL
shSIRT1 + Ad-Null
shSIRT1 + Ad-ATGL
Mander's overlap coefficient (Lyso:Lipid)
8E
GFP-LC3
RFP-LC3
Ad-Null + EX527
Ad-ATGL + EX527

Figure 8. SIRT1 inhibition impairs ATGL-mediated autophagy/lipophagy.

(8A) The induction in autophagy gene expression in response to ATGL overexpression is attenuated with SIRT1 knockdown. Mice were treated with adenoviruses harboring *SIRT1* shRNA, ATGL or their respective controls I week prior to sacrifice (n=5); *P<0.05 vs. Ad-Null and $^{\#}$P<0.01 vs. shSCR. (8B) sh*SIRT1* treated livers had reduced levels of Lamps; a representative western blot from 2 mice is shown. (8C) Western blot showing SIRT1 and ATGL protein levels in hepatocytes treated with sh*SIRT1* and *ATGL* adenoviruses; a representative western blot from 3 hepatocyte experiments is shown. (8D) Colocalization between lysosomes and LDs in hepatocytes treated with dual viruses harboring shSCR/sh*SIRT1* and Ad-Null/Ad-*ATGL* (n=3); p<0.001. (8E) Hepatocytes transfected with dual sensor LC3 and treated with 10 mM EX527, reveal impaired autophagic flux upon SIRT1 inhibition, which blocked the increase in flux following ATGL overexpression (n=3).

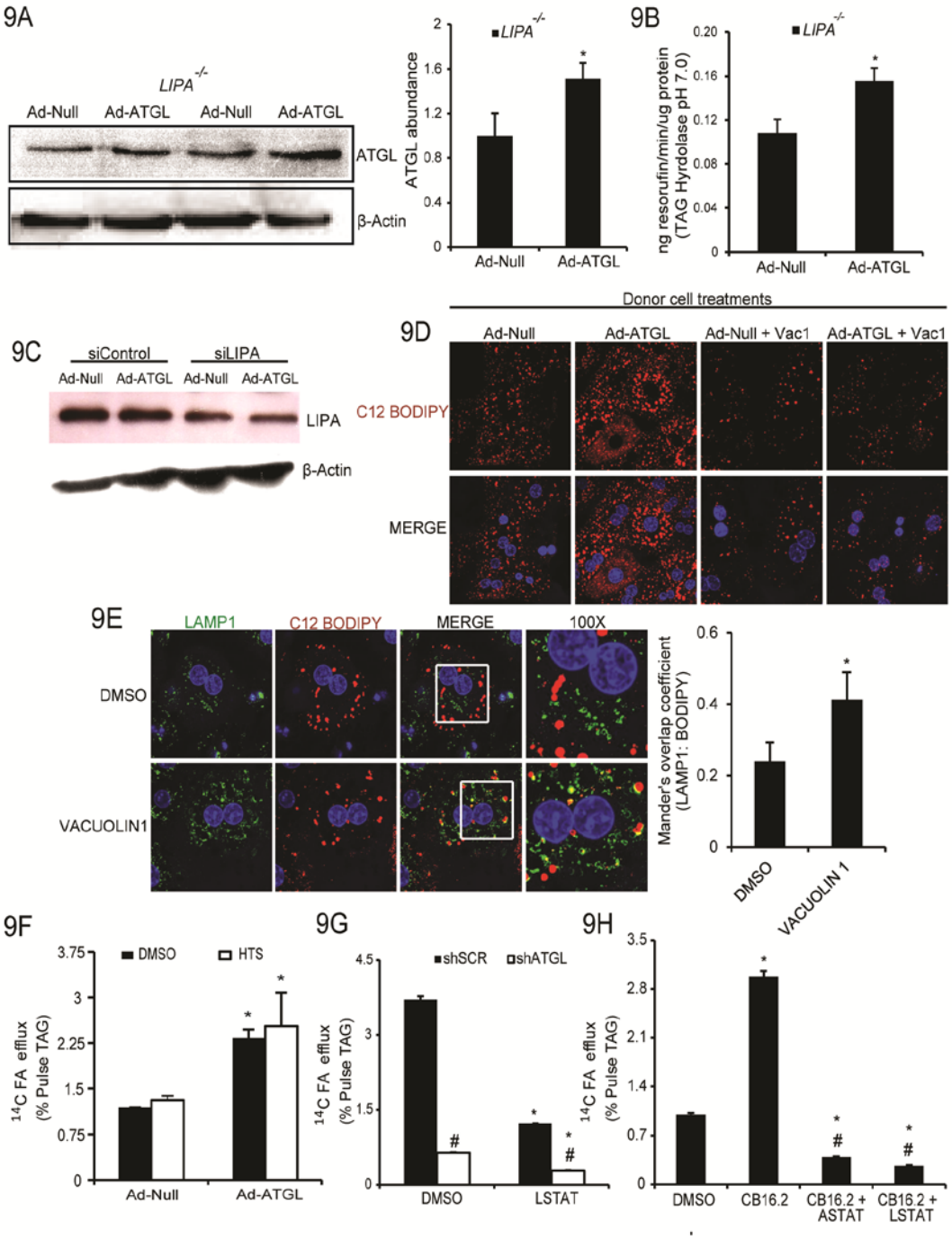
9A
LIPA-/-
Ad-Null
Ad-ATGL
Ad-Null
Ad-ATGL
ATGL
β-Actin
LIPA-/-
ATGL abundance
Ad-Null
Ad-ATGL
9B
LIPA-/-
ng resorufin/min/ug protein (TAG Hyrdolase pH 7.0)
Ad-Null
Ad-ATGL
9C
siControl
siLIPA
Ad-Null
Ad-ATGL
Ad-Null
Ad-ATGL
LIPA
β-Actin
Donor cell treatments
9D
Ad-Null
Ad-ATGL
Ad-Null + Vac1
Ad-ATGL + Vac1
C12 BODIPY
MERGE
9E
LAMP1
C12 BODIPY
MERGE
100X
DMSO
VACUOLIN1
Mander's overlap coefficient (LAMP1: BODIPY)
DMSO
VACUOLIN 1
9F
DMSO
HTS
14C FA efflux (% Pulse TAG)
Ad-Null
Ad-ATGL
9G
shSCR
shATGL
14C FA efflux (% Pulse TAG)
DMSO
LSTAT
9H
14C FA efflux (% Pulse TAG)
DMSO
CB16.2
CB16.2 + ASTAT
CB16.2 + LSTAT

Figure 9. **ATGL-driven FA efflux is mediated via the lysosome.**

(9A) Western blot confirmation of ATGL overexpression in *LIPA*$^{-/-}$ livers. (9B) TAG hydrolase assay in ATGL overexpressing livers from *LIPA*$^{-/-}$ mice. (9C) Efficacy of si*LIPA* in primary mouse hepatocytes 60 hours post transfection; a representative western blot from 2 replicates in shown. (9D) A transwell experiment in primary mouse hepatocytes show that the Ad-ATGL transduced donor cells FA efflux and storage in acceptor cells which is blocked with the addition of 75μM vacuolin 1 (n=3). (9E) Increased C12-BODIPY (558/568) within lysosomes following 75μM vacuolin 1 (n=3). (9F) Addition of 10μM HTS, a pan-FABP inhibitor, did not influence FA efflux (n=3); *P<0.01 vs. Ad-Null. (9G) Chronic inhibition of ATGL using Ad-shATGL in combination with LAListat reduces FA efflux of hydrolyzed FAs. (9H) Addition of ATGListatin or LAListat in the presence of FA reuptake inhibitor CB16.2 reduces the accumulation of FAs in the media (n=3 for 9G and 9H); P<0.005.

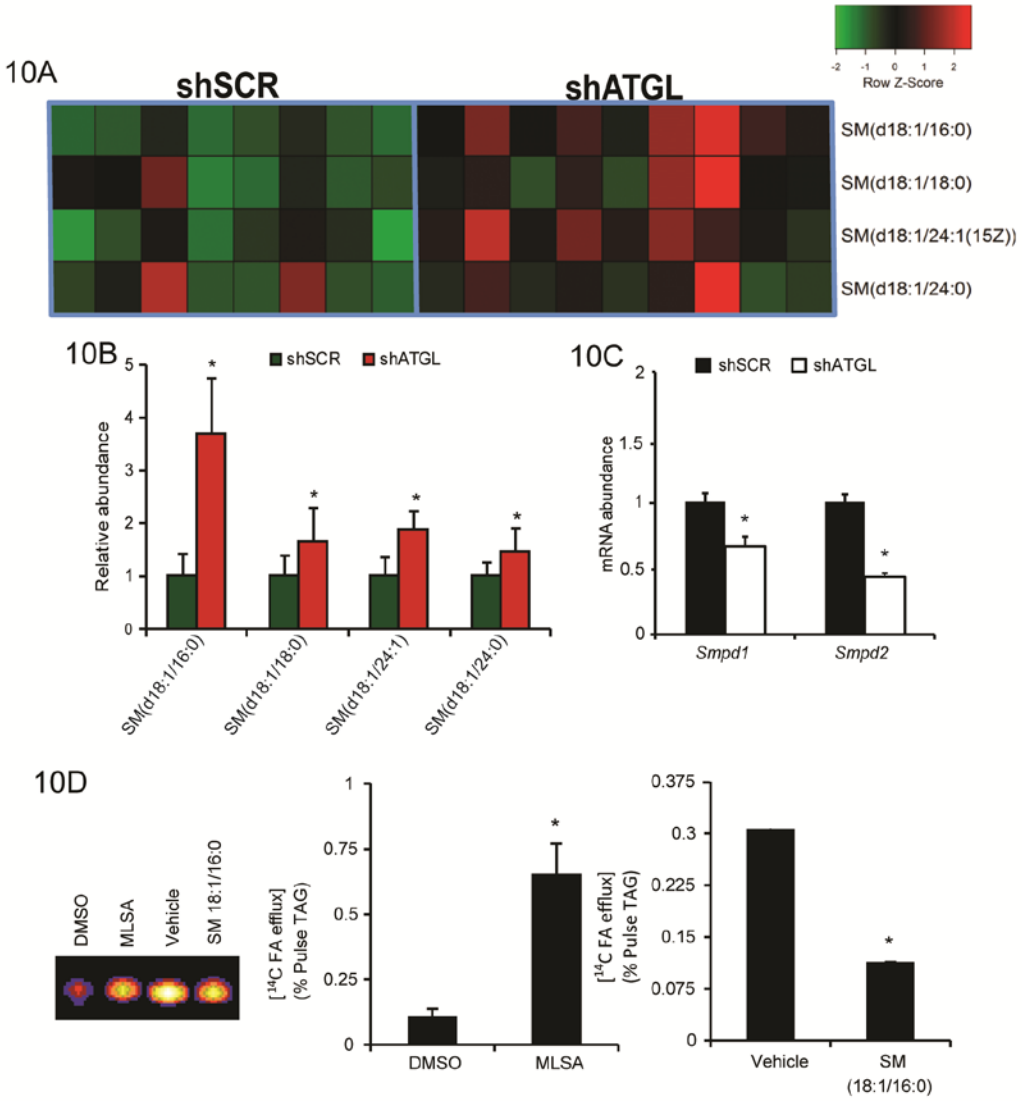
10A
shSCR
shATGL
Row Z-Score
SM(d18:1/16:0)
SM(d18:1/18:0)
SM(d18:1/24:1(15Z))
SM(d18:1/24:0)
10B
shSCR
shATGL
Relative abundance
SM(d18:1/16:0)
SM(d18:1/18:0)
SM(d18:1/24:1)
SM(d18:1/24:0)
10C
shSCR
shATGL
mRNA abundance
Smpd1
Smpd2
10D
DMSO
MLSA
Vehicle
SM 18:1/16:0
[14C FA efflux] (% Pulse TAG)
DMSO
MLSA
[14C FA efflux] (% Pulse TAG)
Vehicle
SM (18:1/16:0)

Figure 10. Sphingomyelins alter lysosomal signaling and subsequent media FA efflux.

(10A-B) Heat maps or individual lipid quantification generated from lipidomic analysis of livers from scrambled control or ATGL knockdown virus treated livers reveal increased sphingomyelin accumulation in shATGL treated livers (n=7); *P<0.05 vs. shSCR. (10C) Lysosomal and golgi-specific genes involved in sphingomyelin hydrolysis are lower in the absence of hepatic ATGL (n=6); *P<0.05 vs. shSCR. (10D) Media FA levels are increased following treatment with MLSA1, a TRPML1 agonist, and reduced following treatment with SM (d18:1/16:0), a TRPML1 antagonist. Representative radiographs of FAs are shown and quantification data are from n=3; *P<0.05.

DISCUSSION

Herein, we define a previously unrecognized interplay between ATGL-catalyzed lipolysis and autophagy. We show that ATGL promotes autophagy/lipophagy, which in turn catalyzes the catabolism of LDs. Additionally, SIRT1 plays a critical role in linking ATGL signaling to the increase in autophagy/lipophagy. Finally, we characterize the importance of lysosomal fusion to the plasma membrane and FA efflux as an important pathway influencing hepatic energy metabolism.

The past decade has seen significant advances in our knowledge about the molecular mechanism of autophagy and its role in the pathophysiology of multiple disorders. Despite a long known linkage between *LIPA* deficiency and steatosis in humans (Burke and Schubert, 1972), lipophagy has only recently been shown to contribute directly to hepatic lipid catabolism (Singh et al., 2009). In addition, recent work has suggested that chaperone-mediated autophagy indirectly contributes to lipophagy in the liver. Specifically, chaperone-mediated degradation of the LD proteins Perilipin 2 and Perilipin 3 promote increased access of ATGL to the LD surface to enhance lipolysis (Kaushik and Cuervo, 2015). The findings in the current study extend these data and suggest that the increase in ATGL activity promotes macro- and microlipophagy, which in turn are responsible for the catabolism of LDs. In addition to ATGL containing LIR motifs (Martinez-Lopez et al., 2016), the studies with ATGListatin indicate that ATGL could influence lipophagy by providing a signal that allows for recognition of LDs

by autophagosomes and lysosomes in addition to the increased lipophagic/autophagic machinery. However, the mechanistic details through which lipophagy works are still being elucidated.

ATGL is recognized to play a central role in lipolysis in many tissues. Upon lipolytic stimulation ATGL and additional lipolytic proteins are recruited to the LD surface to promote TAG hydrolysis (Coleman and Mashek, 2011). Although the hydrolyzed FAs would presumably be available to enter a myriad of potential metabolic pathways, numerous studies have shown that ATGL selectively channels hydrolyzed FAs to oxidative pathways (Ahmadian et al., 2010; Ong et al., 2011; Wu et al., 2012). This selective partitioning of FAs to the mitochondria is reminiscent of starvation-induced autophagy, which supplies cells with substrates to generate energy in times of need. Studies from our laboratory and others have shown that LDs interact with numerous organelles including mitochondria (Khan et al., 2015b; Pu et al., 2011; Wang et al., 2011), which is speculated to facilitate transfer of FAs for their subsequent oxidation (Herms et al., 2015; H. Wang et al., 2011). The data in the current manuscript suggest that FAs, especially in response to ATGL activity, are largely effluxed rather than released intracellularly. Consistent with these data presented, overexpression of ATGL in McArdle-RH7777 cells, a hepatoma cell line, also promotes FA efflux (Reid et al., 2008), while studies in fibroblasts show that the efflux of FAs is mediated via the lysosome (Groener et al., 1996). While we cannot rule out some contribution of intracellular FA flux to the mitochondria directly from LDs

(Rambold et al., 2015) our data clearly support a model where FAs stored in LDs under lipophagy-mediated efflux prior to subsequent oxidation or entrance into other metabolic pathways. Thus, it is plausible that efflux of FAs us a protective mechanism to reduce lipotoxicity or, alternatively, act as a means to redistribute energy to adjacent cells.

Several studies have also demonstrated that SIRT1 is an important regulator of autophagy. SIRT1 deacetylates several mediator proteins of autophagy such as ATG5, ATG7 and ATG8/LC3 (Lee et al., 2008) and regulates the transcriptional control of autophagy via numerous transcription factors and coactivators including PGC-1α and PPARα. Several similarities were observed between the *SIRT1*$^{-/-}$ and *ATG5*$^{-/-}$ mice, especially the accumulation of damaged mitochondria and deficiency in energy metabolism (Lee et al., 2008). Our laboratory has shown that liver-specific deletion of *ATGL* results in lipid accumulation with a concomitant decrease in SIRT1 activity, PPAR-α/PGC-1α target gene expression and downstream mitochondrial biogenesis and β-oxidation (Khan, et al., 2015; Ong et al., 2011). Thus, the *ATGL*$^{-/-}$ model appears similar to the *SIRT1*$^{-/-}$ and *ATG5*$^{-/-}$ mice with alterations in energy metabolism due to reduced mitochondrial capacity. The current experiments bridge these studies to show that SIRT1 mediates the effects of ATGL on autophagy/lipophagy induction. Thus, these studies provide an additional mechanism through which SIRT1 is regulated, in this case via ATGL-catalyzed lipolysis, to influence downstream metabolism.

Our lipidomic analysis shows that ATGL deletion results in sphingomyelin accumulation. These differences in lipids are attributed to altered gene expression of the acid (lysosome-specific) and neutral (Golgi-specific) sphingomyelinase enzymes. Most lysosomal storage diseases are due to the defective activity of various lysosomal enzymes resulting in accumulation of specific lysosomal substrates or impaired lipid trafficking as seen in diseases like Niemann Pick Disease Type A, B and C (Schuchman, 2010; Vitner et al., 2010). Sphingomyelins have been shown to negatively regulate the specific lysosomal Ca^{2+} channel, TRPML1, which is also compromised in lysosomal storage disorders (Kiselyov et al., 2010; Shen et al., 2012). We believe that this accumulation of sphingomyelin within the lysosomes could be contributing to the impaired FA efflux (via TRPML1 inhibition) observed in cells lacking ATGL (Schuchman, 2010; Shen et al., 2012) in addition to the reduced flux of FAs through the lipophagy pathway.

Our data suggest that ATGL is not directly responsible for the mass breakdown of LDs in the liver. Rather, ATGL acts as in important signaling node that promotes autophagy/lipophagy, which in turn catalyzes the majority of LD catabolism. This study also highlights the importance of FA efflux as in intermediate step in the catabolism of lipids in the liver. In sum, we provide new insights into the regulation of hepatic lipid and energy metabolism, which underpins non-alcoholic fatty liver disease and related hepatic diseases.

Author Contributions

Conceptualization, A.S and D.G.M; Methodology, A.S; Investigation, A.S, M.T.M, D.Y., C.C, C.L, N.V, D.K; Validation, A.S; Writing-Original Draft, A.S; Writing-Review & Editing, A.S. and D.G.M; Visualization, A.S; Project and Funding Acquisition, D.G.M; Supervision, D.G.M.

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CHAPTER 4

Conclusions and Future Directions

Aishwarya Sathyanarayan wrote this chapter in its entirety

Non-alcoholic fatty liver disease (NAFLD), representing the hepatic manifestation of the metabolic syndrome, is the most common chronic liver disease worldwide. NAFLD can progress from steatosis (characterized by fat/triacylglycerol (TAG) accumulation in >5% of the parenchymal cell/hepatocytes), to increased inflammation and fibrosis resulting in non-alcoholic steatohepatitis, and finally to cirrhosis leading to hepatocellular carcinoma and liver death (Satapathy and Sanyal, 2015). Although previously under-recognized, the accumulating evidence that suggests progression from NAFLD to NASH puts a large number of patients at risk for chronic and debilitating liver disease (Agrawal and Duseja, n.d.). Statistics show that about 3 million new NAFLD cases are diagnosed in the United States annually with over 2 billion occurrences worldwide (Katsiki et al., 2016; Satapathy and Sanyal, 2015). Furthermore, there are no pharmacological options for NAFLD, and the recommended therapy includes lifestyle modifications through diet reduction, an exercise regimen, and weight loss although the efficacy of this regiment is low (Katsiki et al., 2016). In most cases, the etiology of NAFLD is not completely understood and is thought to precede the development of insulin resistance that often accompanies obesity, dyslipidemia and Type 2 Diabetes, encompassing the metabolic syndrome (Angulo, 2002; Cave et al., 2007; Lonardo et al., 2015). The prevalence of NAFLD is expected to rise and thus represents one of the biggest health care challenges in the foreseeable future (Cave et al., 2007; Katsiki et al., 2016; McCullough, 2004).

Given the prevalence and deleterious effects of NAFLD, it is of paramount importance to advance our understanding of the mechanism involved in hepatic TAG accumulation in order to prevent NAFLD development and progression. Since TAG accumulation in the liver stems from increased TAG synthesis, a large focus of research has been on elucidating the TAG synthesis pathway and its role in NAFLD etiology. However, since the discovery of the enzyme adipose triglyceride lipase (ATGL) and its role in TAG breakdown (Zimmermann et al., 2004), several reports have looked at the contribution of lipolysis in the etiology of NAFLD (Jha et al., 2013; Ong et al., 2011).

Previous work in our laboratory identified that ATGL preferentially channels a fatty acid (FA) produced from lipolysis, which is responsible for the first step in fat breakdown towards β-oxidation in the mitochondria (Ong et al., 2011). Additional work revealed that ATGL directly activates the protein deacetylase Sirtuin 1 (SIRT1), a critical nutrient sensor involved in gene regulation and post-translational modification of several targets (Khan et al., 2015a). Specifically, we show that a FA oleate derived through ATGL-catalyzed lipolysis can directly bind and activate SIRT1 (unpublished). We utilized analytical platforms such as reversed phase high performance liquid chromatography (RP-HPLC) and cyclic dichroism binding assays and found that oleate induced SIRT1 enzyme activity in a dose incremental manner.

This data identifies, for the first time, a novel mechanism by which a lipolytic intermediate positively regulates SIRT1 activity. Oleate is a monounsaturated fatty acid (MUFA) found in abundance in olive oil and nuts, key constituents of the Mediterranean Diet. This diet incorporates healthy eating with portions of veggies, nuts, fruits, a splash of olive oil, and red wine. Research has shown that those who practice the Mediterranean Lifestyle – diet plus ample exercise – have reduced incidence of cardiovascular disease with enhanced cognitive function. We speculate that the above-defined regulation of SIRT1 through increased oleate consumption coupled with fasting/exercise may be the principal mechanisms explaining the health benefits of the Mediterranean Lifestyle and for the first time may provide a biological explanation of how the Mediterranean Lifestyle leads to improved health and longer lifespan.

Since lifestyle changes are already the current standard for NAFLD treatment, this understanding of the role of oleate in mediating NAFLD can be used in treatment guidelines. In 2012 the American Association for the Study of Liver Diseases and the American Gastroenterological Association published their recommendations on NAFLD management supporting the implementation of adequate lifestyle modifications (Chalasani et al., 2012). In this context, a Mediterranean diet in combination with exercise was shown to improve insulin resistance and hepatic steatosis in NAFLD patients (Mahady and George, 2016). Additionally, these beneficial effects were achieved in the absence of sustained weight loss (Ryan et al., 2013).

In addition to the above findings, we show that the key signaling node of ATGL-mediated induction of SIRT1 is also involved in autophagy induction. Specifically, we show that functional autophagy/lipophagy mediates the effects of ATGL on lipid droplet degradation in the liver. Our data shows that ATGL mediates FA efflux via lipophagy and lysosomal fusion to the plasma membrane in cells, before their subsequent fate-either oxidation or re-esterification into TAG stores. These findings support previous research from our lab where we show the liver FA binding protein is not required for ATGL-mediated β-oxidation or PPAR-α expression, suggesting an alternate mechanism to intracellular trafficking to FAs must exist (Ong et al., 2014). Thus in conclusion, we believe our studies have unraveled a unique signaling cascade that have transformed the way we think about hepatic lipid droplet catabolism and how it contributes to oxidative metabolism linking NAFLD and metabolic disease alleviation.

NAFLD was first diagnosed in 1980 (Foster et al., 1980) and 35 years later, it is now recognized as the most common liver disease worldwide. Moreover, NAFLD is a strong determinant for the development of the metabolic syndrome. Previous research highlights that impaired insulin signaling in the adipose tissue results in increased lipolysis and FA release that subsequently enter the liver to promote hepatic TAG synthesis and NAFLD development (Gastaldelli et al., 2007). However, our data now provides evidence for the potential of hepatic ATGL/lipolysis activators as a new class of novel therapeutic entities. Additional research will be required to develop and test small molecule

therapeutics that specifically target and activate ATGL as an effort to provide the foundation for novel future drug discovery efforts in this area.

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